Role of Lipoproteins on Pancreatic Islet Cells Survival, Function and Proliferation

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1 **ABSTRACT (ENGLISH)**

The facts that low plasma concentrations of high density lipoprotein (HDL) is a risk factor for developing type 2 diabetes and that patients with type 2 diabetes have low plasma concentration of HDL provided the main rationales for the studies described in this thesis. The effects of HDL and low density lipoprotein (LDL) on the pancreatic islets are unclear, thus, the overall aims were to investigate whether HDL and LDL play a role in the regulation of pancreatic islet cell apoptosis, proliferation and secretory function.

In the present study, HDL has been identified to play an important role on islet cell survival by decreasing basal islet cells apoptosis. Moreover, HDL protected human and mouse islet cells from IL-1β and glucose induced apoptosis, two factors that are increased in type 2 diabetes. Interestingly, the protective effect of HDL against IL-1β induced β-cell apoptosis was still observed when cells were treated with the delipidated protein moiety of HDL or with apolipoprotein A1 (ApoA1) the main protein component of HDL. Surprisingly, the protective effect of HDL against IL-1β induced β-cell apoptosis was also observed in the absence of the main HDL receptor, SRB1. Intracellularly, HDL decreased mRNA expression of Fas, a cell death receptor, and of inducible nitric oxide synthase (iNOS). Both factors are increased in the presence of IL-1β and are known to be involved in IL-1β induced apoptosis. Moreover, HDL increased FLIP expression, an inhibitory protein of the Fas signaling pathway.

LDL decreased glucose-stimulated insulin secretion of β-cells only in the presence of functional LDL receptor and without affecting insulin expression or production. Thus, cholesterol accumulation may play a crucial role in the defect in glucose-stimulated insulin secretion. Moreover, LDL decreased β-cell proliferation, independently of functional LDL receptor expression. Liraglutide, a GLP-1 analogue, was able to counteract the anti-proliferative effects of LDL.

The present work proposes that HDL plays a protective role on islet cells by decreasing basal apoptosis and protecting from glucose and IL-1β induced apoptosis. This work also suggests that plasma concentrations of LDL should be tightly regulated since prolonged exposure to LDL impairs insulin secretion and decreases islet cell proliferation, deleterious effects that were partially prevented by the GLP-1 analogue liraglutide.

2 ABSTRACT (GERMAN)

Das Wissen, dass tiefe Plasmakonzentrationen von Lipoproteinen hoher Dichte (High density lipoproteins, HDL) ein Risikofaktor für die Entstehung von Diabetes Typ 2 darstellen einerseits und dass andererseits Typ-2-Diabetes-Patienten tiefe HDL-Konzentrationen in ihrem Plasma aufweisen, bildete die Grundlage zu den Experimenten, welche im Rahmen dieser Doktorarbeit durchgeführt wurden. Der Einfluss sowohl des HDLs wie auch des Lipoproteins geringer Dichte (Low density lipoprotein, LDL) auf die Langerhans'schen Inseln ist unklar. Folglich war das Ziel dieser Arbeit zu untersuchen, ob HDL und LDL eine Rolle spielen in der Regulation der Apoptose, Proliferation und Funktion der Langerhans'schen Inselzellen.

In der vorliegenden Arbeit wurde HDL als wichtiger Akteur in der Erhaltung der Inselzellen identifiziert, indem es die basale Apoptoserate senkt. Darüber hinaus schützt HDL menschliche und Maus-Inselzellen vor Apoptose, die durch IL-1β und Glucose (beide Faktoren sind erhöht in Typ 2 Diabetes) induziert wird. Der schützende Effekt von HDL gegen die durch IL-1β induzierte Apoptose der β-Zellen konnte reproduziert werden, wenn die Zellen anstelle von HDL entweder mit der delipidierten Protein-Fraktion von HDL oder mit Apolipoprotein A1 (ApoA1, dem Hauptbestandteil dieser Protein-Fraktion) behandelt wurden. Der protektive Effekt von HDL gegen die IL-1β-induzierte Apoptose in β-Zellen konnte auch in Abwesenheit von SRB1, dem Hauptrezeptor für HDL, nachgewiesen werden. Intrazellulär reduzierte HDL die mRNA-Expression sowohl von Fas, einem Rezeptor der Apoptose-Kaskade, als auch diejenige von iNOS (inducible nitric oxide synthase). Diese beiden Faktoren sind erhöht unter IL-1β und es ist bekannt, dass sie in die IL-1β-induzierte Apoptose involviert sind. Ausserdem erhöht HDL die Expression von FLIP, einem Protein, das hemmend auf den Fas-Signalweg wirkt.

Im Gegensatz dazu verringert LDL die Glukose-Stimulierte Insulin Sekretion in den β-Zellen nur dann, wenn der LDL Rezeptor funktional vorhanden ist. Dabei wird weder die Insulinexpression noch -produktion durch LDL beeinträchtigt. Die Cholesterin-Akkumulation wirkt sich folglich vor allem auf die Sekretion des Insulins nachteilig aus. Überdies vermindert LDL die Proliferation der β-Zellen unabhängig von der Expression des LDL-Rezeptors. Nur Liraglutide, ein GLP-1-Analogon, konnte dem anti-proliferativen Effekt von LDL entgegenwirken.

Die vorliegende Arbeit stellt die These auf, dass HDL einen schützenden Einfluss auf Inselzellen durch die Verminderung der basalen Apoptose ausübt und ausserdem die Inselzellen vor der durch IL-1β induzierten Apoptose schützt. Diese Arbeit legt zudem nahe, dass die Plasma-LDL-Konzentration in engen Grenzen gehalten werden sollte, weil eine längere LDL-Exposition die Insulin-Sekretion beeinträchtigt und die Inselzellproliferation verringert. Diese schädliche Wirkung kann teilweise unterbunden werden durch Behandlung mit dem GLP-1-Analogon Liraglutide.

3 ABBREVIATIONS

ABCA1:	ATP-binding cassette transporter A1
ACAT:	acyl-CoA:cholesterol acyltransferase
acLDL:	acetylated LDL
ADP:	adenosine diphosphate
AMP:	adenosine monophosphate
ApoA1:	apolipoprotein A1
ATP:	adenosine triphosphate
CETP:	cholesteryl ester transfer protein
ECM:	extracellular matrix
ER:	endoplasmic reticulum
ERK:	extracellular signal-regulated kinase
FLIP:	FLICE inhibitory protein
GIP:	glucose-dependent insulinotropic polypeptide
GLP-1:	glucagon like peptide 1
HDL:	high density lipoprotein
HMG-CoA:	3-hydroxyl-3-methyl-glutaryl-CoA
IDL:	intermediate density lipoprotein
IFNγ:	interferon γ
IL-1β:	interleukin-1β
iNOS:	inducible nitric oxide synthase
JNK:	c-Jun NH2 terminal kinase
K_{ATP} channel:	ATP-sensitive K^+ channel
LDL:	low density lipoprotein
LDLR:	low density lipoprotein receptor
lHDL:	lipid moiety of high density lipoprotein
Lira:	liraglutide
LXR:	liver X receptor
MAPK:	mitogen-activated protein kinase
NFκB:	nuclear factor κB
NO:	nitric oxide
oxLDL:	oxidized low density lipoprotein
PCR:	polymerase chain reaction

pHDL:	protein moiety of high density lipoprotein
PKB:	protein kinase B
rHDL:	reconstituted high density lipoprotein
S1P:	sphingosine-1-phosphate
SOCS3:	suppressor of cytokine sigaling 3
SRB1:	scavenger receptor B1
SREBP-1c:	sterol regulatory element binding rotein 1c
TNFα:	tumor necrosis factor α
VLDL:	very low density lipoprotein

4 INTRODUCTION

4.1 Diabetes

4.1.1 The History of Diabetes

Diabetes is an ancient disease and its symptoms, which include excessive drinking of water and frequent urination, were first reported by Egyptians 3500 years ago. Since then, many physicians have remarked on the sweet taste of diabetic's urine. However, the cause of the sugar to show up in the urine of diabetics remained a mystery until 1889, when Joseph Von Mering and Oskar Minkowski conducted pancreatectomy in dogs and found out that the pancreas secretes a substance that affects the metabolism of sugar. In 1910, Sir Edward Albert Sharpey-Schafer suggested that people with diabetes were deficient in a single chemical that was normally produced by the pancreas. He proposed the name *insulin* for this substance, from the Latin *insula* (= island) and in reference to the insulin-producing islets of Langerhans in the pancreas [2]. In 1921, Frederick Grant Banting and Charles Herbert Best repeated the work of Von Mering and Minkowski, went further and could reverse diabetes induced in dogs by pancreatectomy by giving them an extract from the pancreatic islets of Langerhans of healthy dogs [3]. They went on to purify the hormone insulin from bovine pancreases thus resulting in an effective treatment, insulin injections. The first patient was treated in 1922. For this work, Banting and his laboratory director MacLeod received the Nobel Prize in Physiology or Medicine in 1923. The same year, the drug company Eli Lilly and Company was able to produce large quantities of highly refined, 'pure' insulin. Insulin was offered for sale shortly thereafter.

4.1.2 Type 1 and type 2 diabetes

The distinction between what is now known as type 1 diabetes and type 2 diabetes was first clearly made by Sir Harold Percival Himsworth, and published in January 1936. He reasoned that diabetes could be caused not only by a lack of insulin but also by a lack of sensitivity to insulin [2].

Diabetes Mellitus is now defined as a group of metabolic disease characterized by high levels of blood glucose resulting from defects in insulin production, insulin action or both. The chronic hyperglycemia of diabetes is associated with long term damage,

dysfunction, and failure of various organs, especially the eyes, kidneys, nerves, heart, and blood vessels. The majority of cases of diabetes fall into two broad categories: type 1 and type 2 [4]. Type 1 diabetes, presents itself as complete or near complete deficiency of insulin secretion caused by an immune mediated selective destruction of the insulin producing β-cells [5]. Type 1 diabetes accounts for 5-10% of those with diabetes, and often occurs in childhood and adolescence, but can occur at any age. Autoimmune destruction of β-cells has multiple genetic predispositions and is also related to environmental factors that are still poorly defined [6-8].

Type 2 diabetes accounts for 90-95% of those with diabetes. Most of the patients are obese and the disease can be described as a combination of resistance to insulin action and an inadequate compensatory insulin secretory response. Thus, the risk of developing type 2 of diabetes increases with age, obesity, and lack of physical activity. Type 2 diabetes is associated with a strong genetic predisposition. However, the genetics related to type 2 diabetes are complex and not clearly defined [4]. In 2005, an estimated 1.1 million people died from diabetes, and almost half of diabetes deaths occur in people under the age of 70 years. Diabetes causes about 5% of all deaths globally each year. The World Health Organization (WHO) estimates that more than 180 million people worldwide have diabetes. This number is likely to more than double by 2030 (source: World Health Organisation).

4.1.3 Pathogenesis of type 2 diabetes

Type 2 diabetes appears to be a multifactorial disease. It is now commonly accepted that the disease arises when β-cells fail to secrete sufficient amounts of insulin to meet the metabolic demand. An increased metabolic demand due to insulin resistance in several tissues usually precedes the development of hyperglycaemia. There is thus a period of normal or near-normal glycaemia in which pancreatic β-cells compensate for insulin resistance by hyper secretion of insulin. However, this period of β-cell compensation is followed by β-cell failure, in which the pancreas fails to secrete sufficient amounts of insulin. The consequence of β-cell failure, which is due to a loss of β-cell function and mass [9-11], coupled with insulin resistance is the development of type 2 diabetes [12, 13]. Thus, in obese and lean diabetic patients, type 2 diabetes is marked by a decrease in β-cell mass that can no longer

compensates for the increased metabolic load, due to especially obesity associated insulin resistance [14, 15]. Insulin resistance is a condition in which normal amounts of insulin are inadequate to produce a normal insulin response in fat, muscle and liver cells. Insulin resistance in adipocytes results in elevated hydrolysis of stored triglycerides leading to an increase in free fatty acids in the blood plasma. Insulin resistance in muscle cells reduces glucose uptake (and so local storage of glucose as glycogen), whereas insulin resistance in liver cells reduces storage of glycogen, making it unavailable for release into the blood when blood insulin levels fall (normally only when blood glucose levels are low), along with increased gluconeogenesis [16].

4.2 Regulation of pancreatic islet cell mass

4.2.1 Pancreatic islet architecture and composition

The islets of Langerhans, named from the German scientist who first observed and described them in 1869, are scattered throughout the pancreas and constitute about 1-2% of the pancreas. An incompletely defined and variable capsule encloses the islet and partially separates endocrine cells from exocrine cells. Pancreatic islets are highly vascularised mini-organs. Classically, they contain four different cell types: insulin-producing β-cells, glucagon-producing α-cells, somatostatin-producing δ-cells, and pancreatic polypeptide-producing PP cells [17]. A fifth type of cells, grehlin-producing epsilon-cells are present mainly during pancreas development and at birth but their number decline postnatally [18, 19]. Interspecies differences in islet architecture have been proposed. Some reports claim that normal adult murine islets have a β-cell core and a non β-cell mantle, whereas the human islet architecture does not have this core-mantle architecture and β-cells and non β-cells are intermingled. Nevertheless, most recent studies point to a similar sub-organisation of human islets, with multiple entities of sub-islets with a β-cell core (Susanne Boner-Weir & Gordon Weir, personal communication). Another possible species difference between human and rodent islets is the cell composition: the proportion of β-cells in the human islets seems much less (55%) than in murine islets (77%), while the fraction of α-cells appears greater in human islets (35%) than in murine islets (18%) [20-22].

4.2.2 Insulin expression, biosynthesis and secretion

The primary structure of insulin was determined by a British molecular biologist Frederick Sanger. It was the first protein to have its sequence determined and for this work, Sanger was awarded the Nobel Prize in Chemistry in 1958. In 1969, after decades of work, Dorothy Crowfoot Hodgkin determined the tertiary structure of the molecule by means of X-rays diffraction studies and was awarded the Nobel Prize in Chemistry in 1964 for the development of crystallography.

The insulin gene is expressed exclusively in the β-cells of the pancreatic islets. This hormone is released in the blood stream principally in response to elevated glucose levels but also to free fatty acids and amino acids. Glucose metabolism in the β-cells generates intracellular signals which stimulate insulin secretion, insulin mRNA translation and insulin gene transcription. Insulin is synthesized in the β-cell in the form of preproinsulin, which carries additional informations to target the nascent protein chain into the endoplasmatic reticulum (ER). There, after cleavage of the signal peptide, it folds efficiently to assume the native proinsulin structure stabilized by three disulphide bonds. Correctly folded proinsulin is directed to the Golgi apparatus from which it is efficiently sorted into secretory vesicles, where it is converted by proteolytic enzymes to insulin and C-peptide [23].

Glucose induces insulin secretion via a complex pathway. Following the uptake of glucose in the β-cell by the glucose transporter glut2, glycolysis in the cell generates increasing amount of ATP. A rise in the cytosolic ATP/ADP ratio closes the K_{ATP} channel, thus depolarizing the β-cell. This depolarization activates the voltage-gated Ca^{2+} channels and consequently elevates intracellular Ca^{2+}. This promotes the insulin-containing granules translocation and binding to the cellular membrane, finally leading to insulin release [24, 25].

4.2.3 Regulation of pancreatic islet cell mass

In healthy individuals, pancreatic β-cell mass is maintained at an optimal level to maintain normal glycaemia. The adult β-cells are plastic and able to increase their population to adapt to changes in metabolic demand, such as pregnancy and non diabetic obesity [26]. Net changes in β-cell mass are reflective of the amount of growth and probably neogenesis minus the degree of death. In the circumstances of prolonged obesity and insulin resistance, the β-cell works under pressure trying to

meet the increased metabolic demand, and eventually succumbs to a collective number of stresses that lead to an increase in β-cell death, a subsequent reduction in β-cell mass, and the eventual onset of type 2 diabetes [27]. The concept of insufficient β-cell mass as the key factor in the pathogenesis of type 2 diabetes has only recently been widely acknowledged [10, 26]. Postnatal β-cell growth is complex and contributed by several mechanisms, including replication, neogenesis and size [28]. β-cell replication seems to be the primary means of increasing its population (for more details see next section) [29]. β-cell neogenesis describes the formation of new β-cells from the ductal epithelium of the pancreas. Although it is generally accepted that β-cell neogenesis likely occurs, the mechanism is poorly defined [30, 31]. β-cell volume also contributes to β-cell mass, though it is unknown whether a bigger β-cell means a larger capacity to produce, store and release insulin.

4.2.4 Signals inducing and preventing β-cell proliferation

Both the ability to generate and expand large amounts of transplantable β-cells and the capacity to encourage β-cell proliferation in the patient represent potential cures for diabetes. Understanding the basic cell cycle machinery responsible for the replication of β-cell is therefore an important challenge in diabetes research [32]. The signal starting with extracellular mitogens is translated into intracellular mediators, which affect the cell cycle machinery composed of both activators and inhibitors. Cyclin-dependent kinases (CDK) and cyclins are generally responsible for driving the cell cycle forward from G1 to S to G2 and finally to mitosis. Throughout this process, these activating factors are controlled by inhibitors (the CKI and INK families) [33] . The β-cell's decision to proliferate seems to reside with CDK4 and cyclin D2 [34]. The β-cell mitogens include glucose, amino acids, insulin, GLP-1, low levels of IL-1β, gastrin, growth hormone, and IGF-1 and 2 among others [35-37]. The gluco-incretins hormones GIP and GLP-1 are secreted by intestinal endocrine cells and have been studied for many years because of their important effect to potentiate glucose-stimulated insulin secretion. Moreover, both peptides have the potential to stimulate β-cell proliferation and to protect the β-cell from apoptosis [38]. The β-cell replication rate is slow and the proliferation of human islet cells cultured *in vitro* is controversial [39].

4.2.5 Signals inducing and preventing β-cell death

Pathways regulating β-cell turnover are also implicated in β-cell insulin secretory function, thus making β-cell destruction and secretory defect indissociable in the development and progression of type 2 diabetes.

Glucose is the key physiological regulator of insulin secretion. Short-term exposure of β-cells to increasing glucose concentrations induces proliferation in a concentration-dependent manner [40, 41]. However the proliferating capacities of these cells are suppressed following prolonged exposure to increased glucose concentration. One potential mechanism by which glucose regulates β-cell mass could be explained by β-cell production of IL-1β following glucose exposure, and subsequent Fas receptor up-regulation [42]. Interleukin-1β is a prototypic proinflammatory cytokine [43]. Upon activation of the Fas receptor by binding of Fas ligand, the activated receptor induces apoptosis of the activated cell [44]. In the presence of FLIP, a caspase-8 inhibitor, Fas engagement is directed to β-cell proliferation. However excessive glucose stimulation can decrease FLIP and induce Fas receptor death signalling.

IL-1β is a central cytokine regulating β-cell function, viability and replication. At low concentrations IL-1β promotes β-cell function and survival whereas at high concentrations it induces apoptosis. Moreover, IL-1β is found in increased amounts in the β-cells of type 2 diabetic patients [42]. Sustained and intense IL-1 receptor engagement causes progressive functional impairment and is followed by cell death [45, 46]. IL-1β is cytotoxic to rodent β-cells, causing inhibition of glucose-stimulated insulin secretion and expression of the gene Nos2 encoding inducible nitric oxide synthase (iNOS), which leads to nitric oxide (NO) formation and cell death [47]. The key signalling pathways activated by IL-1β in β-cells are the mitogen-activated protein kinase (MAPK) and the nuclear factor kappa B (NFκB) pathways. The MAPKs comprise ERK, p38 and JNK. Their activation is increased in β-cells compared to non β-cells, and this correlates with increased susceptibility to IL-1β toxicity [47]. NFκB comprises a collection of dimers composed of various combinations of members of the Rel family. Five mammalian Rel proteins have been identified: p50, p52, c-Rel, p65 and RelB. Prior to cytokine exposure, NFκB is sequestered in the cytoplasm by binding to inhibitor protein kappa Bα (IκBα). Following cytokine exposure, IκBα is phosphorylated, ubiquitinated and degraded by the proteasomal complex, liberating

NFκB that is then able to translocate to the nucleus and bind κB consensus sequences in promoter regions of numerous proinflammatory genes [48]. IL-1β activates the transcription factor NFκB in rodent and human islet cells, and blocking NFκB activation prevents cytokine-induced apoptosis in these cells. The MAPK and NFκB signalling pathways are not independent and interact together to induce cytokine response [47]. In rodent β-cells, IL-1β induces the expression of iNOS resulting in production of NO. Both ERK and p38 are required for IL-1β-induced iNOS expression and NO production. Moreover, NO contributes to cytokine-induced apoptosis via the activation and stimulation of JNK and p38 MAPK and suppression of Akt [49]. Cytokines, such as IL-1β, either produced by the β-cells themselves in response to hyperglycaemia or by inflammatory cells, which localize at the periphery of the islets, can participate in the induction of apoptosis [50, 51]. Adipokines, such as TNFα, may also have deleterious effects on β-cell survival.

Due to the high secretory demand it endures, the endoplasmatic reticulum (ER) is very well developed and highly active in β-cells. This also likely increases the susceptibility of these cells to ER stressors. However, activation of the unfolded protein response (UPR) in β-cells in response to ER stress may also provide protection to β-cells by attenuating the ability of cytokines such as IL-1β to signal and activate the expression of downstream targets [52-54]. The unfolded protein response (UPR) is a survival mechanism whereby malformed and misfolded proteins forming in the ER set off a response that leads to a down regulation of general protein synthesis. However, if the UPR is strong and extended chronically this can induce ER stress and apoptosis [52, 55].

Increased oxidative metabolism, due to increased metabolic demand, generates reactive oxygen species (ROS) and reactive nitrogen species (RNS) which ultimately activate stress-induced pathways, such as NFκB or stress kinases, to influence cell fate [56, 57].

Each form of stress-induced apoptosis can be prevented or at least markedly reduced by GLP-1 treatment. Studies of the signaling pathways mediating the antiapoptotic actions of GLP-1 support a role for PI3K, Akt, cAMP, and MAPK in part via decreased level of caspase 3 and increased levels of Bcl-2 and Bcl-xl [58-60].

In obesity abnormal lipid deposition is found in many tissues other then adipose tissue, including β-cells. Chronic accumulation of lipid in β-cells causes β-cell

dysfunction and apoptosis [61]. Long-term exposure of β-cells to saturated fatty-acids such as palmitate appears highly toxic, whereas monounsaturated fatty acids such as oleate protect against both palmitate and glucose-induced β-cell apoptosis [62, 63]. Finally, the combination of elevated blood glucose and lipid concentrations is particularly toxic for β-cells [15].

4.3 Lipoproteins

4.3.1 Discovery of lipoproteins

The first inkling that fat transport system existed in the blood of animals can be traced to Boyle in 1665. In 1774, Henon showed that this milky fluid contained fat, and in 1924 Gage and Fish showed that blood taken from humans after a fatty meal contained tiny particles which they named chylomicrons. Cholesterol was first discovered in bile and gallstones by Poulletier de la Salle in 1769 and then rediscovered in 1815 by Chevreul who named it “cholesterine”. Only later was cholesterol found in blood (Boudet 1833). The high density lipoprotein (HDL) was first isolated from horse serum in 1929 by Marcheboeuf and the low density lipoprotein in 1950 by Gofman. Later it was showed that flotation of plasma in the ultracentrifuge revealed an array of lipoproteins that included very low density lipoprotein (VLDL), low density lipoprotein (LDL) and HDL, and permitted quantitation. Free fatty acids were discovered in the plasma by Szent-Gyorgi and Tominaga in 1924 and reinvestigated by Dole, Gordon and Cherkes (1956). They explored the physiological significance of free fatty acids and their binding to albumin and found that they varied with feeding and fasting. From further studies, it was concluded that lipoprotein-bound triglycerides were delivered to adipose cells for uptake after meals; during fasting, the fat cells secreted free fatty acids, which provided fuel for any tissue. The protein components of the lipoproteins were characterized in the period from 1960 to 1970 and the LDL receptor was identified by Goldstein and Brown in 1974 [64].

4.3.2 Lipoproteins structure and metabolism

A lipoprotein is a chemical assembly that contains both proteins and lipids. Cholesterol and triacylglycerol, which are water insoluble, are contained inside a core formed of phospholipids and proteins, thus rendering the molecule water soluble. The

lipid and protein composition varies between the different classes of lipoproteins. The lipoproteins are classified according to their density. Chylomicrons are the smaller and the less dense lipoproteins, followed by very low density lipoproteins (VLDL), low density lipoprotein (LDL) and finally by high density lipoproteins (HDL). The main role of lipoproteins is the transport of cholesterol in the circulation, which is an essential component of cell membranes and steroid hormones.

Cholesterol is derived from both exogenous dietary sources and endogenous biosynthetic pathways. Dietary lipids are predominantly composed of triglycerides, phospholipids and cholesterol. They are taken up from the intestine by the enterocytes which esterifies and assembles them into chylomicrons. Endothelial-bound lipoprotein lipase hydrolyses triglycerides in circulating chylomicrons to generate chylomicrons remnants. The glycerol and free fatty acids generated by the lipoprotein lipase action enter the muscle cells and adipocytes. The triglycerides depleted and thus cholesterol rich chylomicron remnants are taken up by hepatocytes, where they are degraded in the lysosomes. The cholesterol is then exported out of the liver in bile acids and finally excreted. Whereas during fasting, the cholesterol of the hepatocytes that originates from dietary lipids or from liver de novo synthesis is exported in the circulation by VLDL. These lipoprotein particles are of similar protein and lipid composition to chylomicrons but are smaller, synthesized in the liver and carry mainly endogenous lipids. Their function is to transport fatty acids to adipose tissue and muscle. In the circulation, as a result of the lipoprotein lipase (LPL) activity, VLDL are degraded in the same manner as chylomicrons are, thus releasing free fatty acids that are taken up by the muscle cells and the adipocytes. As a result of the large buoyant VLDL degradation, the particles shrink to become the smaller intermediary density lipoprotein (IDL) and then the even smaller LDL. Both the liver and the peripheral tissues can take up LDL. In humans, the majority of serum cholesterol is transported by LDL particles. The liver degrades about 40% to 60% of LDL. The other major player in cholesterol metabolism is HDL. As LPL digests VLDL on the endothelial cell surface, some excess surface material (cholesterol and phospholipids) is transferred to the HDL, which transports excess cellular cholesterol back to the liver [1, 65] (fig 1+2).

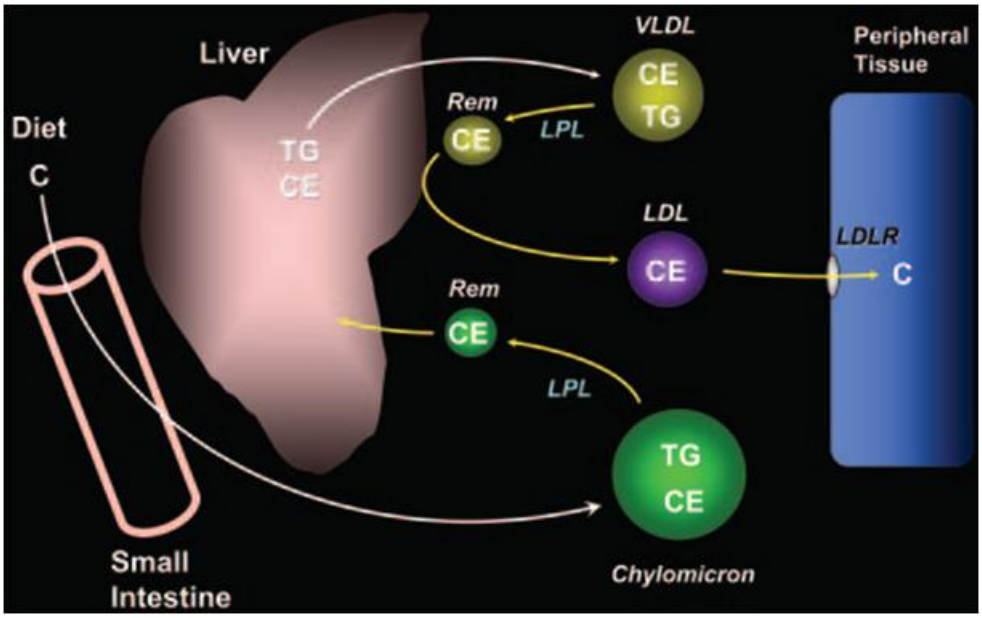


Figure 1: Pathways for cholesterol delivery and production. C indicates free cholesterol; CE, cholesteryl esters; TG, triglycerides; Rem, remnants; LPL, lipoprotein lipase; LDLR, LDL receptor [1].

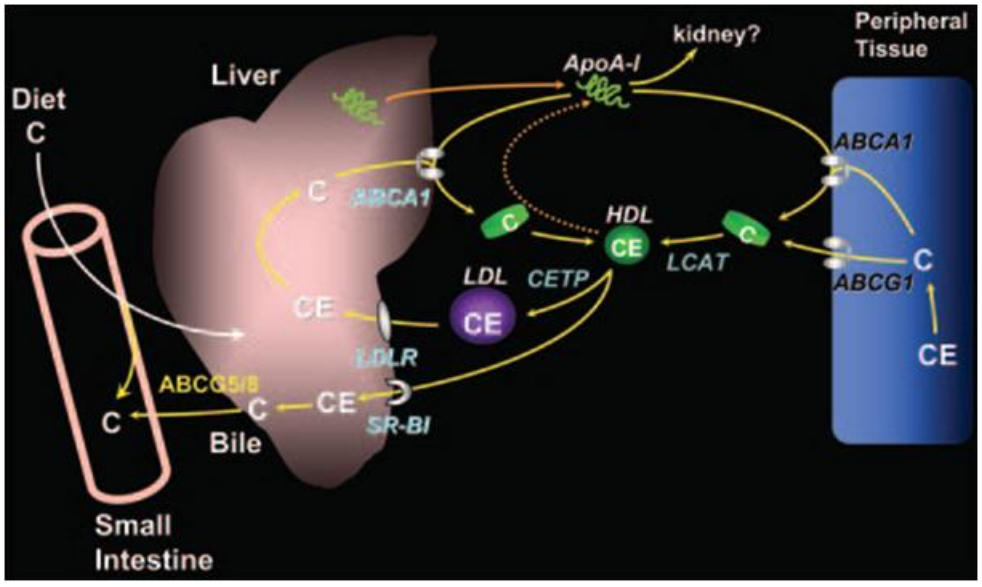


Figure 2: Reverse cholesterol transport. C indicates free cholesterol; CE, cholesteryl esters; LCAT, lysolecithin cholesterol acyltransferase; CETP, cholesteryl ester transfer protein; LDLR, LDL receptor; SR-B1, scavenger receptor B1 [1].

4.4 High density lipoprotein and its receptors

4.4.1 HDL structure and biosynthesis

The HDL fraction in human plasma is heterogeneous in terms of size, shape, composition, and surface charge. The HDL particle can be discoidal or spherical.

Discoidal HDL consists of a bilayer of phospholipids and a small amount of unesterified cholesterol. The hydrophobic phospholipids in the bilayer are surrounded by an annulus of apolipoproteins that renders the particle water-soluble. Most discoidal HDL contains two or three molecules of apolipoprotein-A1 (ApoA1). Spherical HDL contains a hydrophobic core of cholesteryl esters surrounded by a surface monolayer that consists of phospholipids, unesterified cholesterol and apolipoproteins. Lecithin cholesterol acyltransferase (LCAT) rapidly transforms discoidal HDL into spherical HDL [66]. Apolipoproteins are associated with HDL, mainly ApoA1 (70%) and ApoA2 (20%). Other proteins are also found associated with HDL and some of them have enzymatic activities: lecithin cholesterol acyl transferase (LCAT), cholesterol ester transfer protein (CETP), phospholipids transfer protein (PLTP) and paraoxanase [67].

HDL is synthesized through a complex pathway. HDL assembly initially involves the cell surface ATP-binding cassette transporter A1 (ABCA1) mediated transfer of cellular phospholipids and cholesterol to extracellular lipid-poor ApoA1. This is followed by the remodelling in the plasma of HDL particles by the esterification of cholesterol by the enzyme LCAT, the exchange between HDL and other lipoproteins of both apolipoproteins and lipids. Finally the hydrolysis of lipids of HDL is mediated by various lipases and exchange of lipids by the PLTP and CETP. The lipids will then be carried back to the liver by the LDL particles [68]. The liver is the major source of plasma HDL, and intestine is likely to be responsible for the remaining HDL biosynthesis [69].

4.4.2 HDL receptor and signal transduction

The scavenger receptor family contains at least 9 members and was first discovered by Brown and Goldstein, who were developing an in vitro model for the deposition of LDL cholesterol in macrophages. They discovered that acetylation of LDL (acLDL) redirected the uptake of the particle from classic LDL receptor to a novel acLDL receptor. They also showed that these receptors mediated the binding of a remarkably wide variety of ligands [70]. Scavenger receptor class B type 1 (SRB1) was initially identified in a scavenger receptor expression cloning study using acLDL as the ligand [71]. SRB1 is a 509 amino acid long membrane glycoprotein with a molecular mass of 82 kDa. This protein contains a large extracellular domain and two

transmembrane domains with short cytoplasmic amino and carboxy-terminal domains. SRB1 binds a variety of ligands like acLDL, oxLDL, apoptotic cells, native LDL, VLDL and HDL. SRB1 binds HDL with high affinity, with larger spherical HDL binding more tightly than smaller HDL. Lipid-poor ApoA1 is a poor ligand for SRB1. SRB1 mediates both selective uptake of cholesteryl ester and other lipids from HDL and LDL and bidirectional unesterified cholesterol movement [68, 72]. SRB1 is expressed in a variety of tissues including liver, endothelial cells and ovaries. SRB1 is expressed in mouse islets and βTC3 cell, a mouse insulinoma cell line [73]. However, SRB1 is most highly expressed in tissues that are known to be dependent on HDL cholesterol for bile acid synthesis (liver) and steroidogenesis (adrenal gland, ovaries, and testis). SRB1 binds HDL and the core cholesteryl ester is delivered to the plasma membrane without concomitant uptake and degradation of the entire HDL particle. This process is termed the selective uptake pathway. In addition to this process, SRB1 also stimulates the bidirectional flux of free cholesterol between cells and lipoproteins. The function of the receptor in other tissue is unclear as well as its intracellular signal transduction [74]. In healthy endothelial cells, the SRB1 apoptotic pathway, which is caspase-8 mediated, is turned off by eNOS (endothelial nitric oxide synthase) and HDL, thus preventing inappropriate apoptotic damage to the vascular wall. When HDL levels are low, eNOS is relocalized away from SRB1, which turns on SRB1 induced apoptosis [75].

4.4.3 Biological actions of HDL

HDL classically functions in reverse cholesterol transport, removing cholesterol from peripheral tissues and delivering it to the liver and to steroidogenic organs by binding of HDL to the high affinity HDL receptor SRB1. The risk of atherosclerosis, a progressive inflammatory disease characterized by the accumulation of lipids and fibrous elements within the wall of large arteries [76], is inversely related to circulating level of HDL cholesterol. In mouse models of atherosclerosis, ApoA1 and SRB1 provide atheroprotection [77]. The protective nature of HDL has been previously attributed only to its role in reverse cholesterol transport. However, new studies show that alternative mechanisms of action of HDL must be considered. HDL inhibits the chemotaxis of monocytes, the adhesion of leukocytes to the endothelium, endothelial dysfunction, LDL oxidation, stimulates endothelial cell proliferation and inhibits

endothelial cell apoptosis. The latter effect seems to involve ApoA1 and sphingosine-1-phosphate (S1P), the major bioactive lipid of HDL, and cellular protein synthesis but the precise mechanism remains largely unknown [78, 79]. TNFα induced endothelial cell apoptosis is inhibited by HDL, and this is associated with attenuated induction of caspase-3 which is a component of all primary apoptotic pathways [80]. Growth factor deprivation related apoptosis of endothelial cells is also suppressed by HDL [81], and this involves the phosphorylation and activation of protein kinase B (PKB), known to be involved in cell survival [82]. All these recently discovered biological actions of HDL, as well as reverse cholesterol transport, remain to be further studied in order to understand the role of HDL in endothelial cell protection. And this may help to understand the role of HDL on other cell type, where studies are less abundant but could show that HDL affects their function and survival.

4.4.4 ApoA1 structure and biosynthesis

Plasma ApoA1 is a 243-residue protein synthesized primarily in the liver and the intestine [68]. In plasma, ApoA1 cycles between lipid-poor and lipid rich forms. The cycling was described in vitro and follows four different steps: (a) generation of monomolecular lipid-poor ApoA1 from spherical HDL; (b) acquisition by the lipid-poor ApoA1 of phospholipids and unesterified cholesterol from cell membranes and plasma lipoproteins to form discoidal HDL; (c) the conversion of discoidal HDL to spherical HDL; (d) the remodelling of the large spherical HDL by plasma factors in processes that result in the dissociation of monomolecular lipid-poor ApoA1. There are three potential sources of lipid-poor ApoA1 in the plasma: (a) it may be released as lipid-poor protein after its synthesis in the liver and intestine; (b) it may be generated in the plasma during remodelling of spherical HDL; (c) it may be released from triglyceride rich lipoproteins that are undergoing hydrolysis by lipoprotein lipase. Any lipid-poor ApoA1 that is generated in the plasma may either be excreted through the kidney and be irreversibly lost or it may be relipidated and retained in the plasma [66].

4.4.5 ATP-binding cassette transporter A1

ATP-binding cassette transporter (ABC) is a superfamily of membrane transporters containing 48 members grouped into 7 subclasses labelled ABCA through ABCG.

Mutations in ABC genes cause a variety of diseases, including cystic fibrosis, Startgardt's macular degeneration, and disturbances in lipid and lipoprotein metabolism. All ABC transporters use ATP to generate the energy needed to transport metabolites across the membranes. Four members of this large family have been shown to have a major impact on lipoprotein metabolism and cell cholesterol biology: ABCA1, ABCG1, ABCG5 and ABCG8.

ABCA1 is a 2261 amino acid membrane protein. It is predicted to have a N terminus oriented in the cytosol and two large extracellular loops. ABCA1 mediates the transport of cholesterol, phospholipids and other lipophilic molecules across the cellular membranes, where they are removed from cells by lipid-poor HDL apoliproteins. It removes cholesterol that would otherwise accumulate as cytosolic cholesteryl ester droplets. The molecular mechanisms for ABCA1-mediated lipid efflux remains poorly understood. ABCA1 protein level and activity are highly regulated by multiple transcriptional and posttranscriptional processes. Excess intracellular cholesterol is converted to a "second messenger" oxysterol, which regulates ABCA1 through the activation of the nuclear receptor liver X (LXR). Under basal conditions, ABCA1 proteins are highly unstable. The interaction of apoliproteins with ABCA1 reduces the rate of the transporter degradation by inhibiting its proteolysis. Metabolites associated with inflammation and diabetes can destabilize ABCA1. Unsaturated fatty acids, which are elevated in diabetes and the metabolic syndrome, directly destabilize ABCA1 [1, 83, 84].

As nonhepatic cells are unable to degrade cholesterol, ABCA1 plays a critical role in regulating intracellular cholesterol metabolism [85]. Systemic ABCA1 knock-out mice have impaired glucose tolerance but normal insulin sensitivity. ABCA1 is highly expressed in islets β-cells and absence of β-cell ABCA1 results in accumulation of cellular cholesterol, reduction in insulin secretion and a progressive impairment in glucose tolerance. Thus, ABCA1 plays a critical role in β-cell cholesterol homeostasis and is required for proper insulin secretion [86].

ABCA1 is mainly known as a transporter but it also acts as a full ApoA1 receptor that transduces signal from ApoA1 by complexing and activating cdc42, a small G protein, and downstream kinases [87].

4.4.6 Biological actions of ApoA1

ApoA1, as the major protein component of HDL, plays a critical role in reverse cholesterol transport by its interaction in its lipid-poor form with ABCA1. This induces lipid loading of the particle and thus deloading lipid from the cell. Increased plasma concentration of ApoA1 correlates inversely with the incidence of coronary arterial disease [88, 89]. This can be explained by an increase in HDL concentration which implicates an increased reverse cholesterol transport. However other mechanisms are possibly also responsible of the decrease of the incidence of coronary arterial disease. ApoA1 has been shown to protect endothelial cells from apoptosis by a yet unknown mechanism. Moreover, ApoA1 mimetic peptides reduce the inflammation markers induced by endotoxin in endothelial cells [90].

Type 2 diabetes patients have a reduced circulating HDL concentration which corresponds to a decrease in ApoA1 concentration, due to an elevated clearance of HDL molecule and simultaneously of ApoA1 [91, 92]. Glucose inhibits and insulin stimulates the expression of endogenous ApoA1 mRNA in human hepatic cells [93]. Thus, the reduced ApoA1 plasma concentration in type 2 diabetes seems to be a consequence of increased clearance of HDL and ApoA1 and decreased mRNA ApoA1 expression [94], due to elevated glucose concentration and reduced insulin concentrations or insulin sensitivity. ApoA1 positively modulates glucose homeostasis, by stimulating the phosphorylation of AMP kinase and acetyl-coenzyme A carboxylase and thus elevating glucose uptake in myocytes [95]. Through this mechanism, ApoA1 stimulates glucose utilisation and improves insulin resistance in peripheral tissues. In this regard, ApoA1 seems to be an important target for the prevention and therapy of type 2 diabetes.

4.4.7 Sphingosine-1-phosphate structure and biosynthesis

About 10 years ago, sphingosine-1-phosphate-like (S1P-like) lipids were shown for the first time to be associated with plasma lipoproteins [96]. One of these S1P-like lipids was then identified as being S1P. S1P concentration per unit amount of protein in lipoprotein fractions is mostly in HDL, then LDL and VLDL [97]. S1P is derived from shingosine, which was named in 1884 after the Greek mythological creature, the Sphinx, because of its enigmatic nature. Sphingolipids form a class of lipids characterized by a particular aliphatic aminoalcohol which is sphingosine.

Sphingosine can be released from ceramides, a process catalyzed by the enzyme ceramidase. S1P belongs to a class of lipid mediators that function not only inside the cell but also as ligands for specific cell surface receptors. S1P levels in cells are low and tightly regulated by the balance between its synthesis and degradation. The activity of the sphingosine kinase, which catalyses the ATP-dependent phosphorylation of sphingosine, is stimulated by many agonists indicating that it is a central regulating enzyme of S1P. The degradation of S1P is mediated by 2 different pathways: one is the reversible dephosphorylation back to sphingosine by S1P lyase and the second is the irreversible degradation by a S1P lyase [98].

4.4.8 Sphingosine-1-phosphate receptors and signal transduction

To date five S1P receptors have been identified: $S1P_1$, $S1P_2$, $S1P_3$, $S1P_4$, $S1P_5$. They are members of the superfamily of G protein coupled receptor (GPCR). S1P receptors consist of an extracellular N-terminal domain, 7 transmembrane domains and an intracellular C-terminal domain. $S1P_1$, $S1P_2$ and $S1P_3$ are widely expressed in various tissues whereas the expression of $S1P_4$ is confined to lymphoid and hematopoietic tissue and $S1P_5$ to the central nervous system. All five receptors from this family bind to S1P with high affinity except for the $S1P_4$ which binds preferentially phytosphingosine-1-phosphate. Binding of S1P to its receptors activates different signaling pathways via the heterotrimeric G proteins. $S1P_1$ couples exclusively to G_i whereas $S1P_2$ and $S1P_3$ couples to G_i, G_q and G_{13}, and $S1P_5$ couples to $G_{i/o}$ and G_{12}. The coupling of the different receptors with different G proteins can activate many different downstream proteins, such as ERK, PLC, PI3K and Akt or inhibit the adenylate cyclase leading to a decrease in cAMP production. S1P receptors differentially regulate the small GTPase of the Rho family (Rho, Rac, cdc42) [98, 99].

4.4.9 Biological actions of sphingosine-1-phosphate

S1P acts as an intracellular second messenger as well as an extracellular ligand for the S1P receptors. Diverse external stimuli, particularly growth and survival factors and chemoattractants, stimulate the S1P kinase to generate intracellular S1P. However, the targets of the intracellular S1P are not defined so far. S1P precursors, ceramide and sphingosine, have been associated with growth arrest and cell death [100, 101]. By contrast, S1P enhances growth and survival in different cell type [102].

As these metabolites are interconvertible, it is probably not their absolute amounts, but rather their relative levels, that determine cell fate. Through the activation of different signaling pathways, S1P can regulate many different cellular functions, such as adherens junction assembly, cytoskeletal changes, migration, proliferation and apoptosis. S1P mediates the lipoprotein-induced cytoprotective actions through the S1P receptors and their intracellular signalling pathways [103].

Four S1P receptors ($S1P_1$, $S1P_2$, $S1P_3$ and $S1P_4$) are present on mouse and rat islets as well as on the rat insulinoma cell line INS-1 [104]. S1P acting on S1P receptors coupled to G_q mediates protective effects on islet β-cells against cytokine-induced apoptosis [105].

4.5 LDL and its receptors

4.5.1 LDL structure and biosynthesis

LDL is the major carrier of cholesterol in plasma. LDL has a highly-hydrophobic core consisting of esterified cholesterol. This core is surrounded by a shell of phospholipids and unesterified cholesterol as well as one single molecule of apoliprotein-B100 (ApoB100). ApoB100 is synthesized in the liver and intestine. LDL is formed from the lysis and digestion of VLDL in the circulation by LPL. LDL particles vary in size and density [65]. A higher concentration of small dense LDLs, which are more prone to modifications such as glycosylation and oxidation, is now recognized as a risk factor for developing cardiovascular diseases. As well, these small dense LDL particles are more present in patients suffering from type 2 diabetes [106].

4.5.2 LDL receptors and signal transduction

LDL is recognized by a variety of receptors, depending also if the particle is under its native form or whether it is modified. They are members of either the LDL receptor family or the scavenger receptor family. The LDL receptor (LDLR), the founding member of the family, was the first to be discovered and has been extensively studied [107]. It is a membrane-spanning glycoprotein with a highly conserved structure in human and other species. The LDLR is synthesized in the rough endoplasmic reticulum as a precursor protein. Following the maturation process in the Golgi apparatus, the mature form of the receptor contains 839 amino-acid

residues with a molecular mass of 160 kDa, with 5 functional domains. The LDLR mediates the clearance of LDL particles from plasma. The mature LDL receptor proteins are guided to the cell surface, where they cluster into the coated pits on the cell membrane. Upon binding, the LDL-LDLR complex is taken up by the cells via clathrin-mediated endocytosis. In endosomes, the ligand dissociates from the receptor due to the local low pH. After dissociation, the LDLR recycles back to the cell surface. The LDL particle is targeted to the lysosomes where cholesterol is made available by hydrolysis of the cholesteryl esters. The free cholesterol released has the following effects: incorporation into membranes, inhibition of the synthesis of new LDLR, inhibition of cholesterol synthesis by reducing the synthesis of HMGCoA reductase and promotion of the activity of ACAT (acly CoA:cholesterol acyl transferase) which synthesizes cholesterol esters [108, 109]. These regulatory events are mediated by sterol regulatory element binding protein (SREBP) which monitors the free cholesterol concentration in the cell and adjusts the expression of the cholesterol regulatory genes [110]. LDLR transcription is controlled not only by cholesterol and its derivatives but also by cytokines, growth factors, hormones and secondary messengers. Insulin up regulates the transcription of LDLR [111, 112]. Mutations of LDLR gene underlie the lipid disorder familial hypercholesterolemia.
The second member of the family cloned was the LDL receptor related protein (LRP1) [113]. LRP1 is a large endocytotic receptor widely expressed in several tissues and known to function in areas as diverse as lipoprotein transport, regulation of cell surface protease activity, and control of cellular entry of bacterial toxins and viruses [114-116] . Beside LRP1, apoER2 and VLDL receptor have been recognized as signal transducers, and binding of LDL. LDLR, LRP, VLDLR and apoER2 are expressed on mRNA and protein levels in mouse islets and the β-cell line, βTC3 [73].

4.5.3 Biological actions of LDL

The major role of LDL is to deliver cholesterol to the tissues, especially to the liver and to steroidogenic cells. However, most of the LDL in circulation, around 70%, is taken up by the liver to be degraded. The liver either further degrades the cholesterol and extracts it from the body in the bile or recycles it and packs it for the circulation into VLDL. As mentioned above, LDL, especially when present in its small dense form, can be modified. Cholesterol accumulation in macrophages, as a consequence

of uncontrolled uptake of modified LDL particles by scavenger receptors (SR-A and CD36), leads to foam cells formation thereby contributing to the initiation and progression of atherosclerotic plaques. Modified LDL, and specifically oxidized LDL, has a detrimental effect on endothelial cells as well as on islet cells [117, 118]. Diabetic LDL triggers apoptosis in vascular endothelial cells [119].

4.6 Lipoproteins in diabetes

Patients with type 2 diabetes have a dyslipidemia characterized by low plasma levels of HDL, increased level of serum triglycerides and free fatty acids. This so-called diabetic dyslipidemia is pro-atherogenic and can in part explain the marked increase in the risk of premature coronary heart disease in type 2 diabetes. In fact, the dyslipidemia might precede the manifestation of type 2 diabetes. This provides one explanation why many patients with type 2 diabetes have already manifested atherosclerothic diseases at the time point of diagnosis of diabetes [120-123].

The triglyceride level is related to an increased hepatic production of VLDL and a reduction of VLDL catabolism and a decreased HDL concentration due to an accelerate HDL catabolism. The VLDL overproduction seems to be mainly due to hepatic resistance to the inhibitory effect of insulin on VLDL production. Although plasma LDL level is usually normal in type 2 diabetic patients, LDL particles show kinetic abnormalities, such as reduced turn-over, which is potentially harmful. Type 2 diabetic patients have an increased level of small dense LDL which are more prone to modifications such as oxidation or glycation [124]. Type 2 diabetes is associated with decreased plasma HDL, due to increased catabolism of HDL particles. Moreover, HDL particles in type 2 diabetes have qualitative abnormalities, such as enrichment in triglycerides and glycation of ApoA1 [124].

Finally, low HDL is a risk factor for developing type 2 diabetes [125] and subjects with predominance of small dense LDL have a greater than two-fold risk for developing type 2 diabetes [126].

4.7 Role of lipoproteins and cholesterol in islets

4.7.1 Cholesterol and islets

Recent evidence suggests that alterations of plasma and islet cholesterol level may contribute to islet dysfunction and loss of insulin secretion. ABCA1, which regulates islet cholesterol efflux, is essential for normal β-cell function, and absence of ABCA1 results in islet cholesterol overload and impaired insulin release [86]. Cholesterol impairs β-cell function and glucose-stimulated insulin secretion. Conversely, lowering β-cell cholesterol levels with drugs increases insulin secretion [127]. Increasing islet cholesterol leads to reduced β-cell function and in some studies increased β-cell apoptosis [73, 127]. The most plausible hypothesis for cholesterol impaired insulin secretion is that, while cholesterol is critical for granule fusion, excess cholesterol causes dysregulation of raft formation and impairs exocytosis. Finally, several genetic alterations of cholesterol metabolism are associated with type 2 diabetes [128].

4.7.2 Lipoproteins and islets

Functional lipoprotein receptors are present in mouse pancreatic islets and in βTC3, a transformed-insulin secreting cell line. β-cells express the LDL receptor, which is responsible for the uptake of LDL particles and their subsequent lysosomal degradation [73, 129]. Islets from LDL receptor -/- mice display markedly decreased LDL uptake compared with islets from wild type mice, thus concluding for a predominant role for LDL receptor in LDL uptake. However, in these LDL receptor -/- islets low levels of LDL uptake are still observed. This can be explained by the presence on the islets of ApoER2 [73], a receptor that can bind LDL with lower affinity [130]. SRB1 is also expressed on mouse islets and could explain the uptake of HDL particles by the islet cells. The role of VLDL and LDL in the apoptosis of islet cells is controversial. In one study, VLDL and LDL caused apoptosis in a time and concentration dependent manner [73]. A second study demonstrates that LDL causes death of islet β-cells by its cellular uptake and subsequent oxidative modification. However, the cell death caused by LDL was claimed to be necrosis and not apoptosis. In the same study VLDL protects islet cells from the LDL induced death [131]. Only oxidised LDL and not native LDL causes apoptosis in islet cells in a third study, and this effect seems to be mediated by JNK [118]. LDL decreases

insulin gene expression only at high concentration, whereas oxLDL decreases it already with low concentration [73, 118]. In all studies investigating the role of lipoproteins on islet cells, HDL consistently affects islet cell survival and secretory function positively. In a study published by Roehrich and colleagues, HDL efficiently antagonized the proapoptotic effects of serum deprivation and incubation with cytokines (cocktail of IL-1β + TNFα + IFNγ) or elevated LDL. This protective effect seems mediated by Akt, since incubation with HDL leads to increased Akt phosphorylation. Finally, Abderrahmani's and colleagues describe that HDL counteracts the deleterious effect of oxLDL on islet cells [118].

4.8 Aim of the study

Type 2 diabetes is characterized by a decrease in β-cell mass that can no longer compensate for the metabolic load created by insulin resistance. Along with hyperglycaemia, patients with type 2 diabetes typically display elevated free fatty acids and triglyceride concentrations, and a decrease in HDL plasma concentration. Moreover, low HDL plasma concentration is a risk factor for developing type 2 diabetes. Preliminary data in rodent β-cell have indicated a role for lipoproteins in the regulation of cell survival. However, the precise role of HDL and LDL on human and mouse β-cell survival, proliferation and secretory function remains to be investigated.
The aim of my thesis is to determine the role of HDL and LDL on mouse and human β-cell apoptosis, proliferation and secretory function, to investigate the putative protective role of HDL on islet cells in the context of type 2 diabetes, and to uncover the mechanisms of action of HDL and LDL in islet cells. Elucidating this may lead to knowledge on the mechanisms underlying the crucial role of lipoproteins in the development and progression of type 2 diabetes.

5 RESULTS

5.1 Role of HDL on islet cells function, survival and apoptosis

5.1.1 HDL and β-cells function

To examine the effect of HDL on mouse and human islet β-cell function, islets were cultured for 4 days in the presence of various concentrations of HDL. Glucose-stimulated insulin secretion in mouse and human β-cells was not affected by the presence of HDL (fig 3A-C).

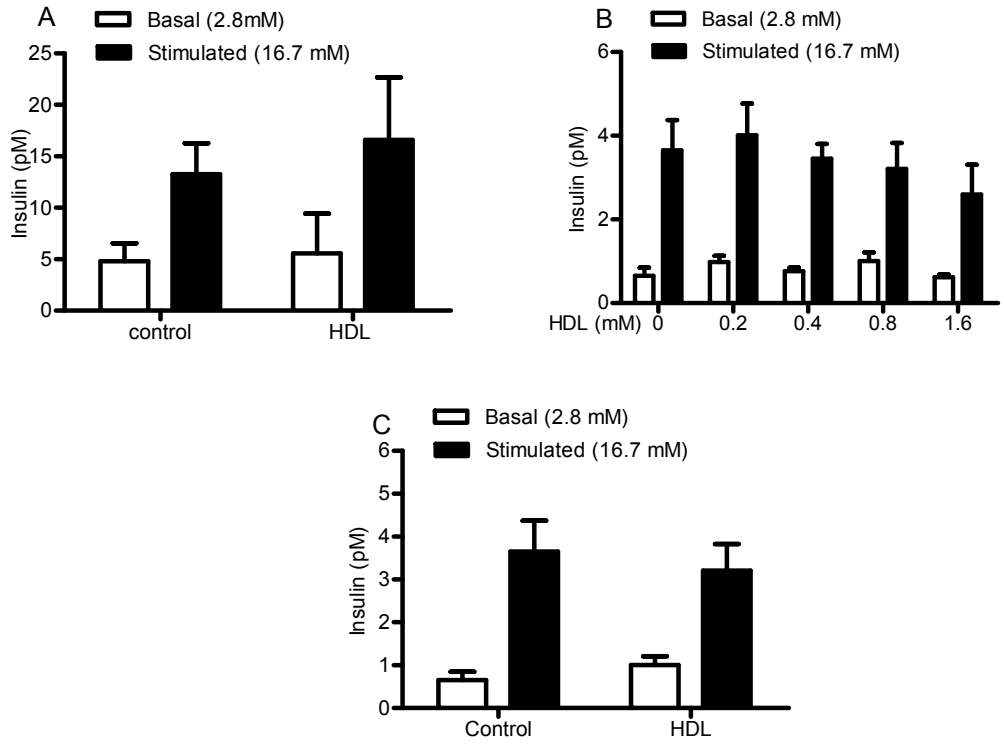


Figure 3: HDL and basal human and mouse β-cell secretory function. Human and mouse islets were cultured for 4 days on extracellular matrix-coated dishes in the absence (control) or presence of HDL (human: 1 mM; mouse: 0.8mM). A: Glucose-stimulated insulin secretion in human β-cells (n=5). B: Glucose-stimulated insulin secretion in mouse β-cells (n=5). C: Glucose-stimulated insulin secretion in mouse β-cells (n=5).

5.1.2 HDL and islet cells proliferation

The role of HDL on basal human and mouse islet cells proliferation was evaluated by incubating human or mouse islets for 4 days with various concentrations of HDL. Proliferation of human and mouse islet cells was not influenced by the presence of HDL (fig 4A-C).

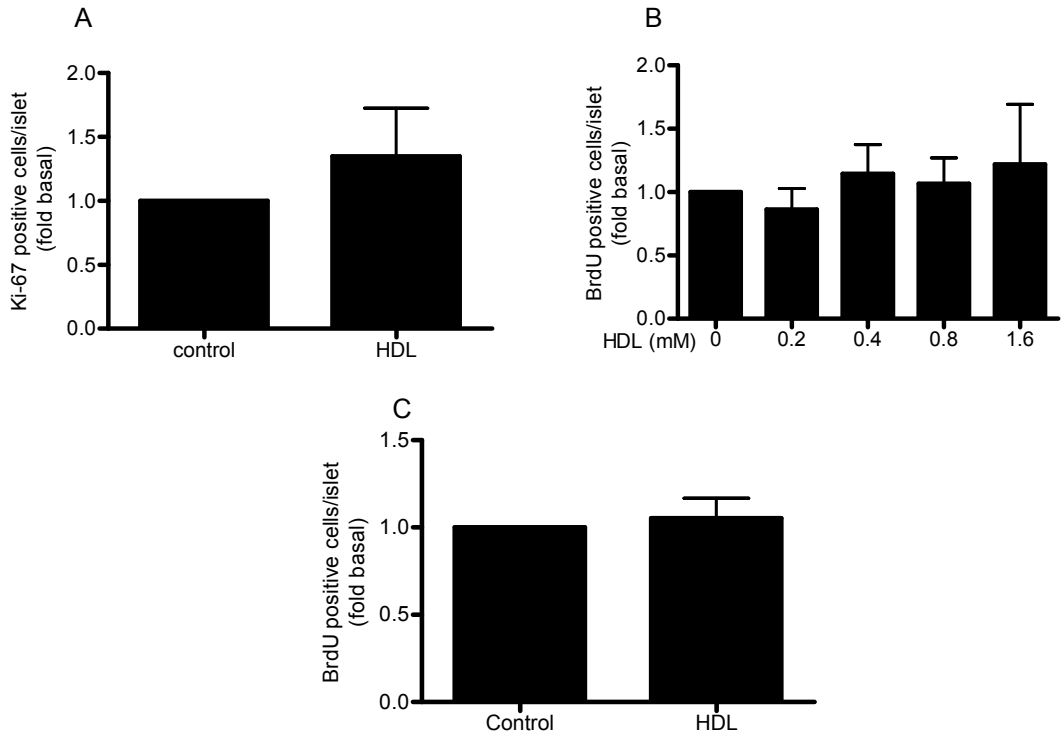


Figure 4: HDL and basal human and mouse islet cells proliferation. Human and mouse islets were cultured for 4 days on extracellular matrix-coated dishes in the absence (control) or presence of HDL (human: 1 mM; mouse: 0.8mM). A. Ki-67 positive human islet cells per islet (n=12), control (absolute value): 0.2 positive cells/islet. B: BrdU positive mouse islet cells per islet (n=5), control (absolute value) 5 positive cells/islet. C: BrdU positive mouse islet cells per islet (n=11), control (absolute value) 3 positive cells/islet.

5.1.3 HDL and islet cells apoptosis

The role of HDL on basal human and mouse islet cells apoptosis was evaluated by incubating human or mouse islets for 4 days with various concentrations of HDL. Exposure of human islets to 1 mM HDL induced a 1.5 fold decrease in islet cells apoptosis (fig 5A). There was a tendency for HDL to decrease basal mouse islet cells apoptosis, in particular at 0.8 and 1.6 mM, however without reaching statistical

significance (fig 5B). Exposure to 0.8 mM HDL induced a 1.5 fold decrease in mouse islet cells apoptosis (fig. 5C), similarly to human islets. The basal rate of apoptosis after 2 days only tended towards a decrease in the presence of 0.8 mM HDL (fig 5D).

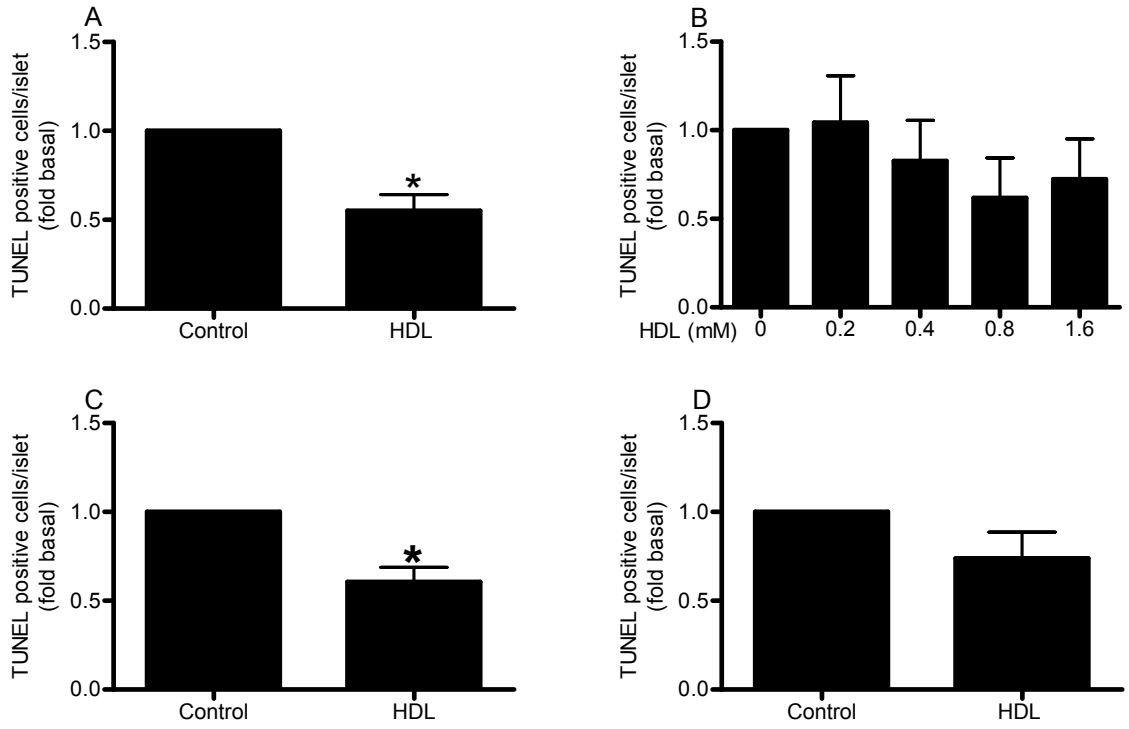


Figure 5: HDL decreases basal human and mouse islet cells apoptosis. Human and mouse islets were cultured in the absence (control) or the presence of HDL (human: 1 mM; mouse: 0.8 mM) for 2 (D) or 4 (A, B, C) days on ECM-coated dishes. A: TUNEL positive human islet cells per islet (n=11), control (absolute value): 0.23 positive cells/islet. B: TUNEL positive mouse islet cells per islet (n=5) control (absolute value): 1.6 positive cells/islet. C and D: TUNEL positive mouse islet cells per islet after 4 days (C, n=17) and 2 days (D, n=15), control (absolute value): 0.75 positive cells/islet (C), 0.52 positive cells/islet (D). * represents $p<0.05$ by Student's t test.

5.1.4 HDL and islets in the presence of elevated glucose concentration

Given the decrease in basal islet cells apoptosis by HDL, we hypothesized that HDL may protect human and mouse islet cells from the deleterious effects provoked by a prolonged exposure to elevated glucose concentration. Human and mouse islets were cultured for 4 days in the presence of elevated glucose concentration and 0.8 mM (mouse) or 1 mM (human) HDL. 33 mM glucose induced a 2 fold increase in mouse and human islet cells apoptosis. Interestingly, HDL was able to reverse

apoptosis induced by the presence of elevated glucose concentrations in both species (fig 6A+B). When the glucose concentration was increased to 22 mM, the apoptosis induced was milder and thus the protective effect of HDL as well (fig 6C). After 4 days, glucose induced a 2 fold decrease in islet cells proliferation and this was not reversed by HDL, as shown in fig 7A for human islets and in fig 7C for mouse islets. In addition, HDL was unable to restore β-cell glucose-stimulated insulin secretion impaired by a prolonged incubation with elevated glucose concentration (fig 7B+D).

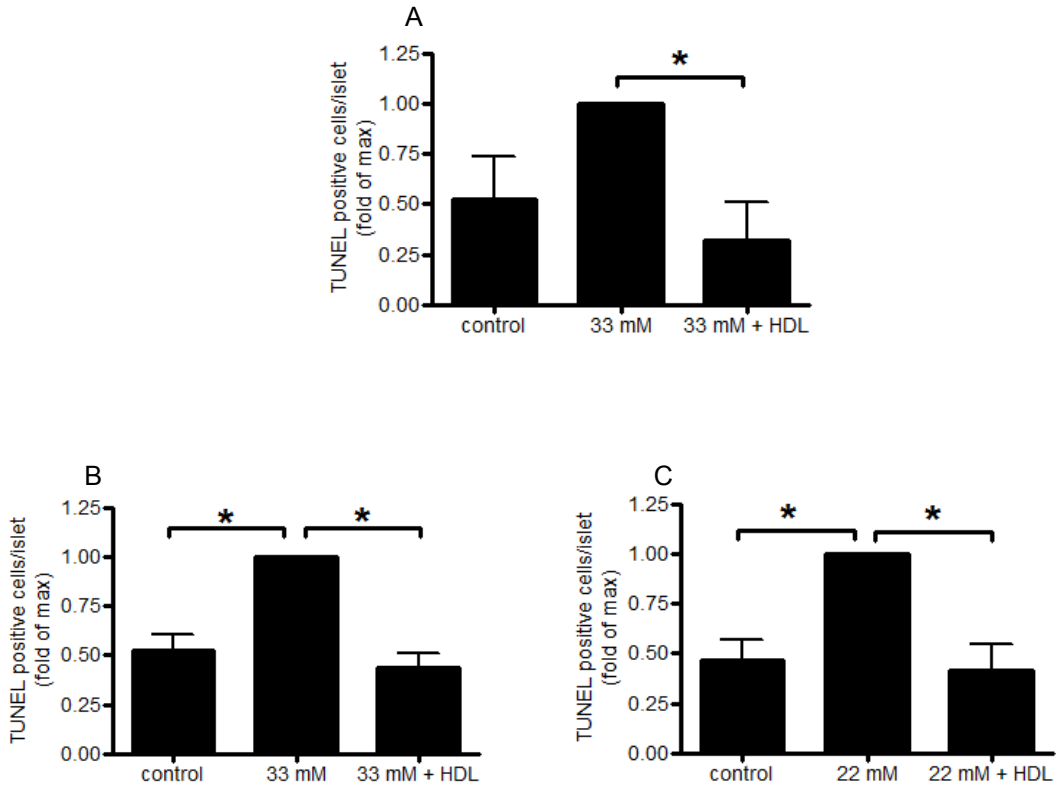


Figure 6: HDL protects human and mouse islet cells from glucose induced apoptosis. Human islets were cultured for 4 days on ECM-coated dishes in 5 (control) or 33 mM glucose in the absence (control) or presence of HDL (1 mM). Mouse islets were cultured for 4 days on ECM coated dishes in 11 (control), 22 or 33 mM glucose in the absence (control) or presence of HDL (0.8 mM). A: TUNEL positive human islet cells per islet (n=4), control: (absolute value) 0.11 positive cells/islet. B: TUNEL positive mouse islet cells per islet (n=7), control: (absolute value) 0.38 positive cells/islet. C: TUNEL positive mouse islet cells per islet (n=6), control: (absolute value) 0.62 positive cells/islet. Where * represents $p<0.05$ versus 33 mM as tested by ANOVA followed by Bonferroni's post-hoc test.

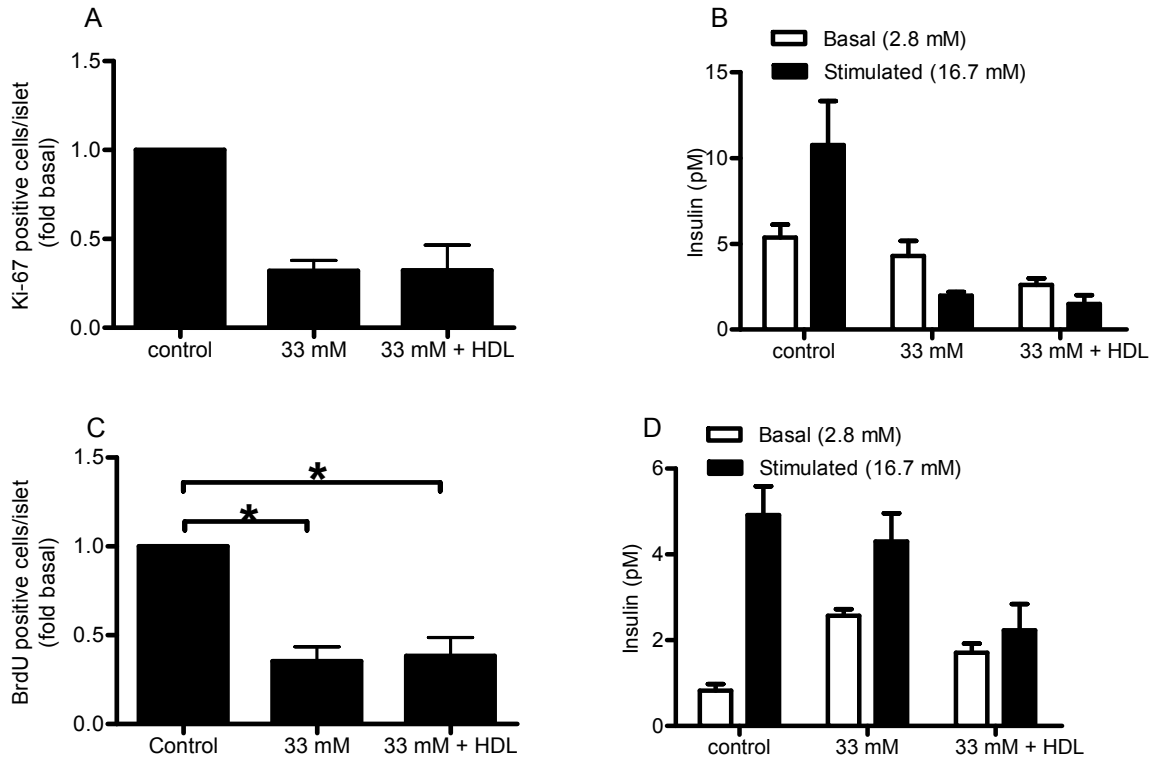


Figure 7: HDL does not restore the impaired secretory function and the reduction in proliferation induced by elevated glucose. Human and mouse islets were cultured for 4 days on ECM-coated dishes in 5 (control human), 11 (control mouse) or 33 mM glucose in the absence (control) or presence of HDL (1 mM and 0.8 mM respectively). A: Ki-67 positive human islet cells per islet (n=2), control (absolute value): 0.46 positive cells/islet. B: Glucose-stimulated insulin secretion in human β-cells (n=3). C: BrdU positive mouse islet cells per islet (n=5), control (absolute value): 4.89 positive cells/islet. D: Glucose- stimulated insulin secretion in mouse β-cells (n=3). Where * represents $p<0.05$ tested by ANOVA followed by Bonferroni's post-hoc test.

5.1.5 HDL and islets in the presence of IL-1β

Since HDL appeared to protect islet cells from glucose-induced apoptosis, we hypothesized that HDL may also protect mouse islet cells from IL-1β induced apoptosis, since it has been proposed to mediate gluco-toxic effects. In order to test this hypothesis, mouse islets were incubated for 2 or 4 days with IL-1β in the presence or absence of HDL. Indeed, HDL protected mouse islet cells from IL-1β induced apoptosis (fig 8A). The HDL protection of islet cells against IL-1β was not effective against impaired glucose-stimulated insulin secretion or decreased proliferation (fig 8B+C).

To determine which islet cell type HDL protected against IL-1β induced apoptosis, islets exposed for 2 days to IL-1β alone or with HDL were analysed for apoptosis by confocal microscopy. This method allowed us to identify apoptotic β-cells (insulin positive) and non-β-cells (insulin negative). In fact, HDL protected mouse β-cells from IL-1β induced apoptosis (fig 9A+B).

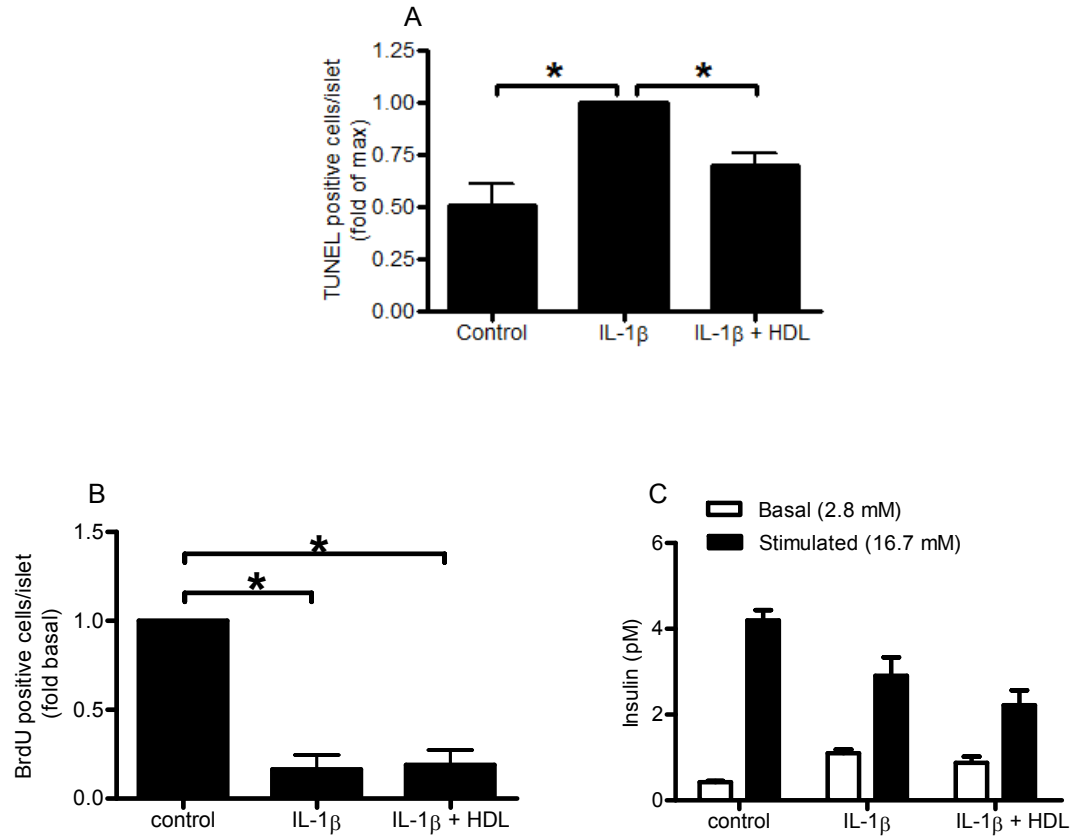


Figure 8: HDL protects mouse islets from IL-1β induced apoptosis. Mouse islets were cultured for 2 (A) or 4 days (B, C) on ECM coated-dishes in the absence (control) or presence of IL-1β (2 ng/ml) and in the absence (control) or presence of HDL (0.8 mM). A: TUNEL positive mouse islet cells per islet after 2 days (n=16), control (absolute value): 0.46 positive cells/islet. B: BrdU positive mouse islet cells per islet after 4 days (n=3), control (absolute value): 3.36 positive cells/islet. C: Glucose-stimulated insulin secretion in mouse β-cells after 4 days (n=3). Where * represents $p<0.05$ as tested by ANOVA followed by Bonferroni's post-hoc test.

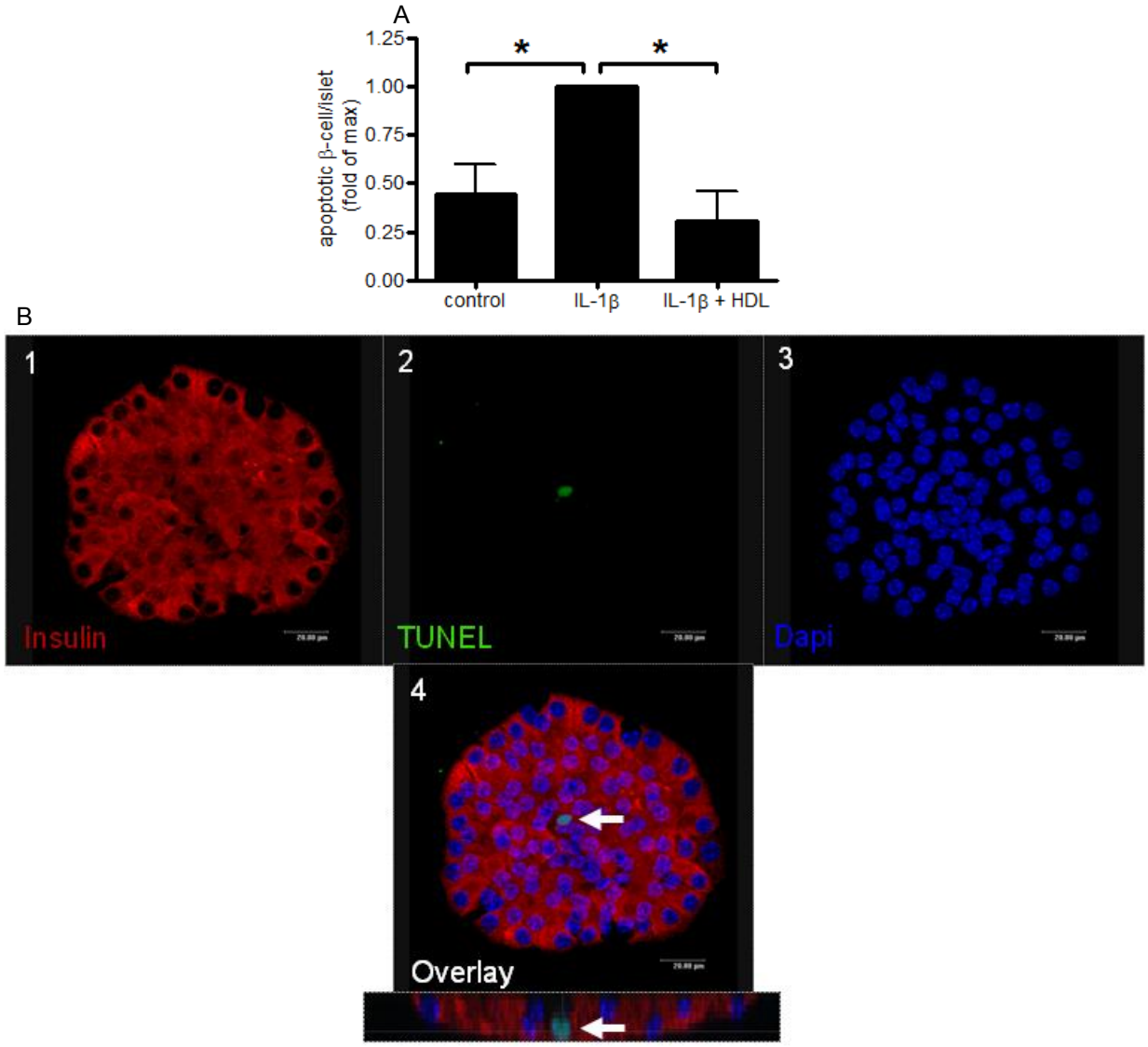


Figure 9: HDL protects mouse β-cells from IL-1β induced apoptosis. Mouse islets were cultured for 2 days on ECM-coated dishes in the absence (control) or presence of IL-1β (2 ng/ml) and in the absence (control) of presence of HDL (0.8 mM). A: TUNEL positive β-cell per islet (n=4), control (absolute value): 0.32 positive cells/islet. B: Confocal microscope pictures of a representative islet co-stained for insulin (1), TUNEL (2) and Dapi (3). Pictures of the different stainings and the overlay (4) are shown. Where * represents $p<0.05$ versus IL-1β as tested by ANOVA followed by Bonferroni's post-hoc test.

5.1.6 Receptor expression

In order to evaluate the expression of the two receptors, SRB1 and ABCA1, a conventional PCR was performed on cDNA obtained from Min6 cells, mouse and human islets and on cDNA from purified human β-cells with primers specific for these

receptors. ABCA1 and SRB1 transcripts could be detected in cDNA samples obtained from mouse islets (fig 10A). These 2 receptors are also present at the mRNA level in Min6 cells (fig 10B), and in human islets (fig 10C). In purified human β-cells, ABCA1 and SRB1 also seem to be present at mRNA level. However, the weak signal observed with the PCR performed on RNA obtained from purified human β-cells could also result from a contamination of the β-cell fraction from the non β-cell fraction (fig 10D).

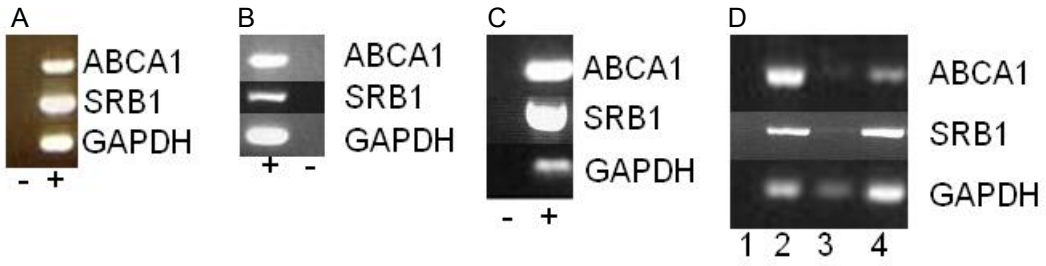


Figure 10: HDL receptor mRNA expression in human and mouse islet cells. Mouse islets, human islets, and purified human β-cells were cultured for 4 days on ECM-coated dishes and RNA was extracted. Conventional PCR with specific primers was performed to detect the expression of the different receptors. A: Mouse islets. B: Min6 cells. C: Human islets. D: Purified human β-cells. (+) and (-) represent cDNA and RT negative control respectively. (1): negative control; (2) total islet cells; (3): β-cell fraction; (4): non-β-cell fraction

5.1.7 Cell line

Given the difficulties and variabilities of working with primary mouse and human islets, the murine cell lines Min6 and INS1 were tested in order to be used as working models for the protective effects of HDL. After 2 days incubation, HDL decreased basal apoptosis in Min6 cells and INS1 cells (fig 11A+12A), similarly to what was observed previously with mouse and human islet cells. However, HDL failed to protect Min6 and INS1 cells from IL-1β (fig 11B+12B) or glucose induced apoptosis (fig 12C). Due to the discrepancies between the results in primary cells and cell lines, only primary cells were used hereafter.

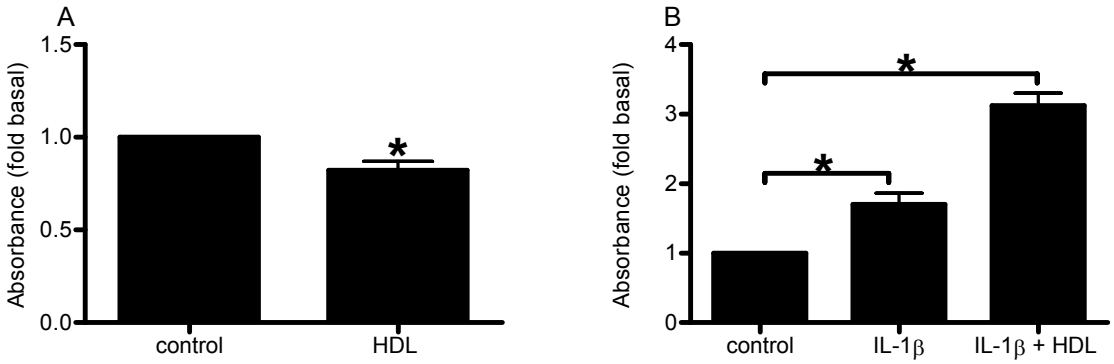


Figure 11: HDL does not protect Min6 cells from IL-1β induced apoptosis. Min6 cells were cultured for 2 days in the absence (control) or presence of HDL (0.8 mM) and in the absence (control) or presence of IL-1β (2 ng/ml). A: Apoptosis in Min6 cells, relative to the absorbance (n=6). A: Apoptosis in Min6 cells, relative to the absorbance (n=6). * represents $p<0.05$ by Student's t test (A) or by ANOVA followed by Bonferonni's post-hoc test (B).

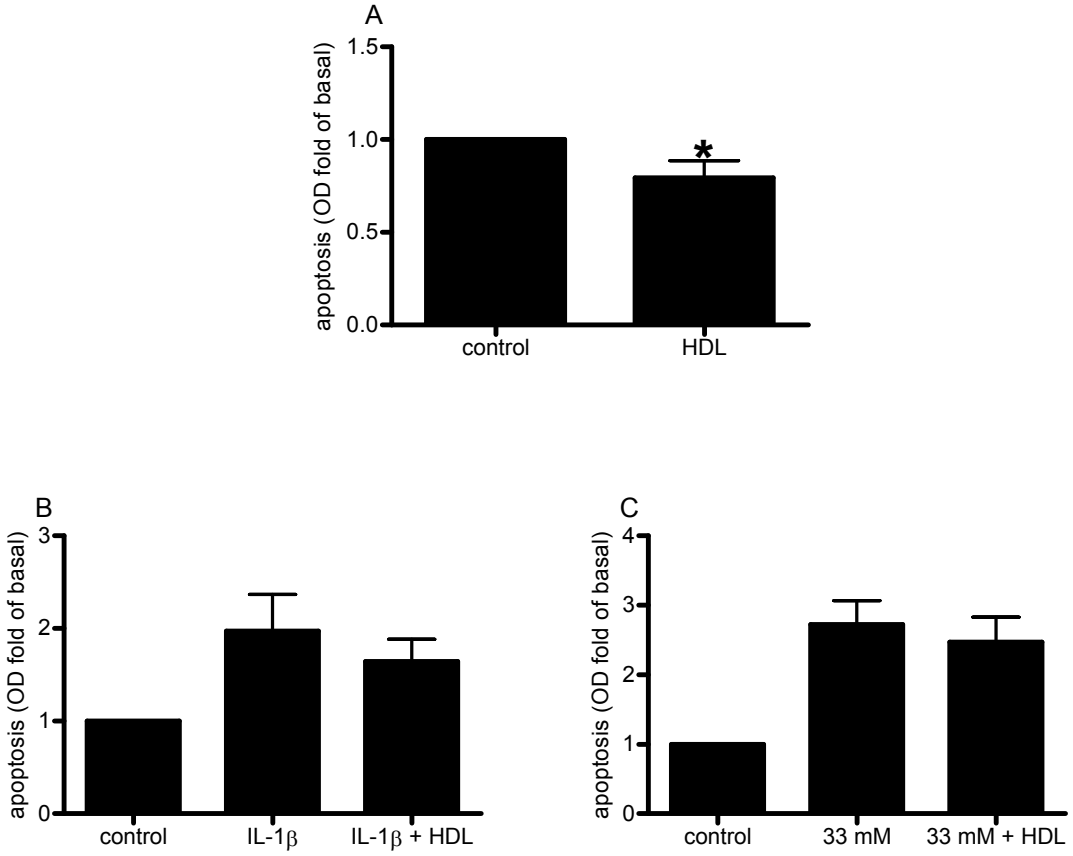


Figure 12: HDL does not protect INS1 cells from IL-1β induced apoptosis. INS1 cells were cultured for 2 days in the absence (control) or presence of HDL (0.8 mM) and in the absence (control) or presence of IL-1β (2 ng/ml) or glucose (33 mM). A: Apoptosis in INS1 cells, relative to the absorbance (n=8). B:

Apoptosis in INS1 cells, relative to the absorbance (n=8). C: Apoptosis in INS1 cells, relative to the absorbance (n=3). * represents $p<0.05$ by Student's t test (A).

5.1.8 HDL protein and lipid moieties and mouse islet cells apoptosis

In order to identify which component of the HDL particle mediated the protective effect against IL-1β and glucose induced apoptosis, HDL was delipidated. Mouse islets were treated with either the delipidated protein moiety (pHDL) or the protein relipidated with phosphatidylcholine and cholesterol, which formed so called reconstituted HDL (rHDL), in the presence of elevated glucose concentration. Similarly to HDL, the HDL protein moiety, whether under its pure protein form or reconstituted with defined lipids, tended to decrease glucose induced apoptosis after 4 days incubation (fig 13A+B). Since this protein moiety is composed of over 90% ApoA1, mouse islets were treated with purified ApoA1. Glucose and IL-1β induced apoptosis was decreased by ApoA1, however only the latter reached statistical significance (fig 13C+D).

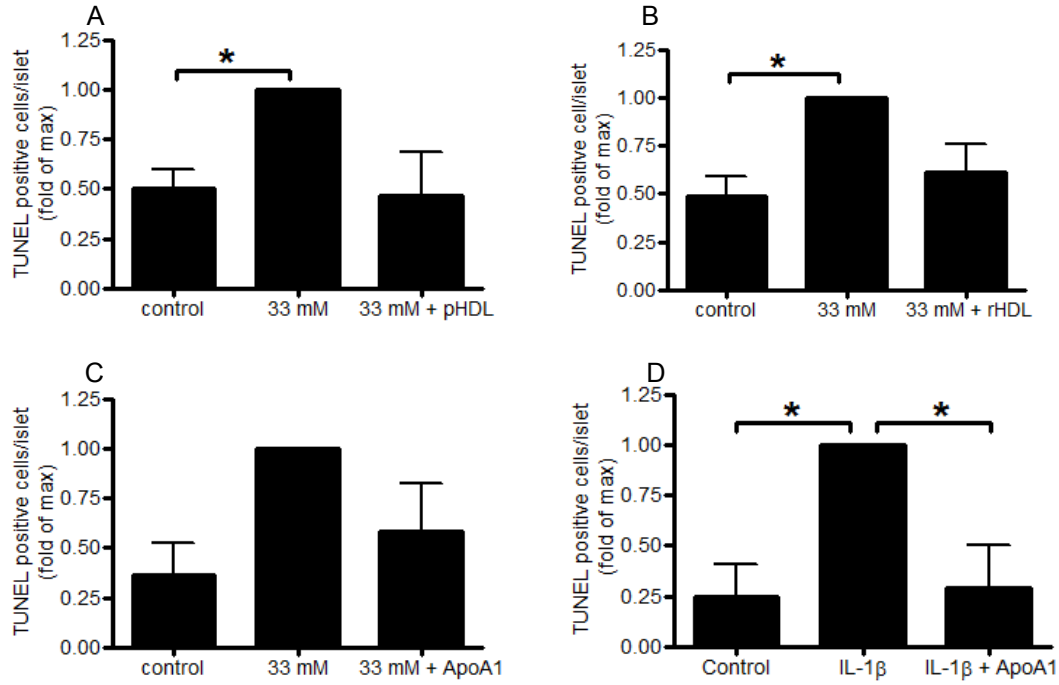


Figure 13: ApoA1 mediates the protective effect of HDL on islet cell apoptosis. Mouse islets were cultured for 2 (D) or 4 (A - C) days on ECM-coated dishes. A: TUNEL positive mouse islet cells per islet after 4 days culture in 11 (control) or 33 mM glucose in the absence (control) or presence of HDL protein moiety (pHDL 20 ug/ml) (n=3), control (absolute value): 0.35 positive cells/islet. B: TUNEL

positive mouse islet cells per islet after 4 days culture in 11 (control) or 33 mM glucose in the absence (control) or presence of reconstituted HDL (rHDL 20 ug/ml) (n=3), control (absolute value): 0.41 positive cells/islet. C: TUNEL positive mouse islet cells per islet after 4 days culture in 11 (control) or 33 mM glucose in the absence (control) or presence of ApoA1 (20 ug/ml) (n=3), control (absolute value): 0.17 positive cells/islet. D: TUNEL positive mouse islet cells per islet after 2 days culture in 11 mM glucose in the absence (control) or presence of IL-1β (2 ng/ml) and in the absence (control) or presence of ApoA1 (20 ug/ml) (n=3), control (absolute value): 0.48 positive cells/islet. Where * represents $p<0.05$ as tested by ANOVA followed by Bonferonni's post-hoc test.

Surprisingly, the lipid moiety of HDL, obtained from HDL lipid extraction and consecutive reloading of the lipid extracted on bovine serum albumin (BSA), also protected islet cells from IL-1β induced apoptosis (fig 14A). IL-1β in the presence or absence of BSA induced islet cells apoptosis (data not shown). However, sphingosine-1-phosphate, a sphingolipid abundant in HDL lipid part that has been shown to exert a protective role on endothelial cells and proposed to mediate the effect of HDL [103], was unable to protect mouse islet cells from IL-1β induced apoptosis at low concentration (fig 14B).

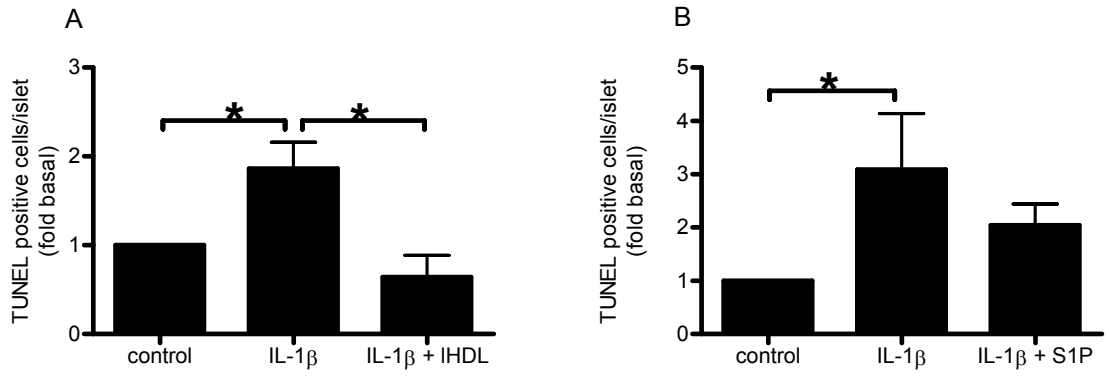


Figure 14: At low concentration, sphingosine-1-phosphate does not protect islet cells from IL-β induced apoptosis. Mouse islets were cultured for 2 days on ECM-coated dishes. A: TUNEL positive mouse islet cells per islet after 2 days culture in 11 mM glucose in the absence (control) or presence of IL-1β (2 ng/ml) and in the absence (control) or presence of HDL lipid moiety (lHDL 0.8 mM) (n=4), control (absolute value): 0.61 positive cells/islet. B: TUNEL positive mouse islet cells per islet after 2 days culture in 11 mM glucose in the absence (control) or presence of IL-1β (2 ng/ml) and in the absence (control) or presence of S1P (100 nM) loaded on BSA (n=4), control (absolute value): 0.43 positive cells/islet. Where * represents $p<0.05$ as tested by ANOVA followed by Bonferonni's post-hoc test.

5.1.9 Mechanisms of the protective effects of HDL

Next, the underlying mechanisms of the anti-apoptotic effect of HDL were studied. Incubation of mouse islets with 0.8 mM HDL for 2 days strongly decreased basal iNOS mRNA expression (fig 15A). IL-1β induced iNOS mRNA expression in mouse islets, but not as much when HDL was present (fig 15B). After 4 days incubation HDL modulated the expression of Fas and FLIP, two molecules involved in the extrinsic apoptotic pathway. Indeed, HDL induced a 1.5 fold down-regulation of the death receptor Fas whereas it causes a 1.5 fold up-regulation of FLIP, an inhibitory protein of the Fas pathway (fig 16A+B). The expression of SOCS3 and Bcl2 was not modulated by the presence of HDL (fig 16C+D).

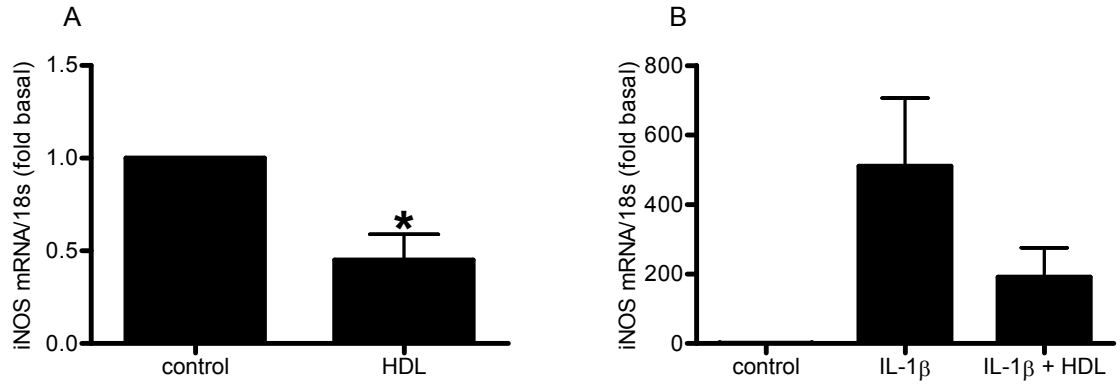


Figure 15: HDL down regulates iNOS mRNA expression. Mouse islets were cultured for 2 days on ECM-coated dishes prior to RNA extraction. Semi-quantitative analysis of gene expression was performed with the Taq-Man technology. A: iNOS mRNA expression after 2 days incubation in the absence (control) or presence of HDL (0.8 mM) (n=5). B: iNOS mRNA expression after 2 days incubation in the absence (control) or presence of HDL (0.8 mM) and in the absence (control) or presence of IL-1β (2 ng/ml) (n=5). * represents $p<0.05$ by Student's t test (A) or by ANOVA followed by Bonferonni's post-hoc test (B).

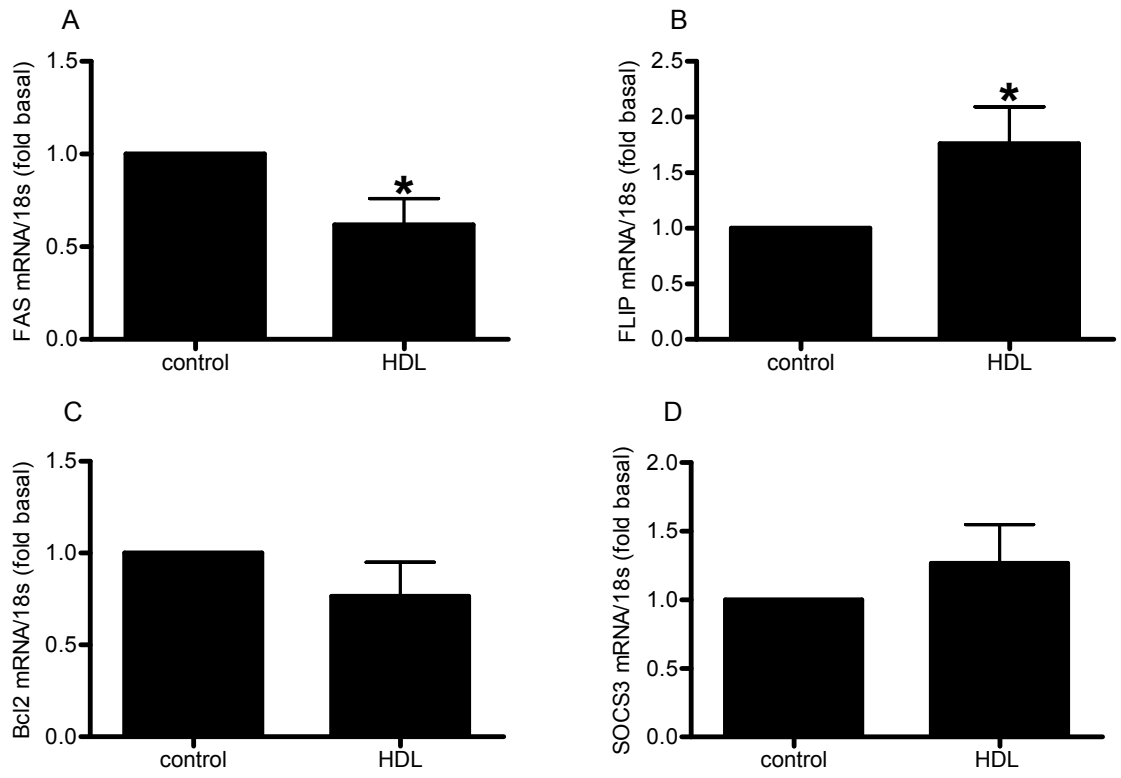


Figure 16: HDL down regulates Fas und upregulates FLIP mRNA expression. Mouse islets were cultured for 4 days on ECM-coated dishes prior to RNA extraction. Semi-quantitative analysis of gene expression was performed with the Taq-Man technology. A: Fas mRNA expression after 4 days incubation in the absence (control) or presence of HDL (0.8 mM) (n=7). B: FLIP mRNA expression after 4 days incubation in the absence (control) or presence of HDL (0.8 mM) (n=6). C: Bcl2 mRNA expression after 4 days incubation in the absence (control) or presence of HDL (0.8 mM) (n=8). D: SOCS3 mRNA expression after 4 days incubation in the absence (control) or presence of HDL (0.8 mM) (n=5). * represents $p<0.05$ by Student's t test.

Upstream of gene expression, IL-1β modulated phosphorylation of enzymes, and specifically MAP kinases. Incubation of mouse islets for 30 min with IL-1β increases the phosphorylation of JNK, ERK and p38, without influencing the amount of each protein (blot not shown). The increased phosphorylation of p38 induced by IL-1β is lower when HDL is added thereafter to the islets (fig 17).

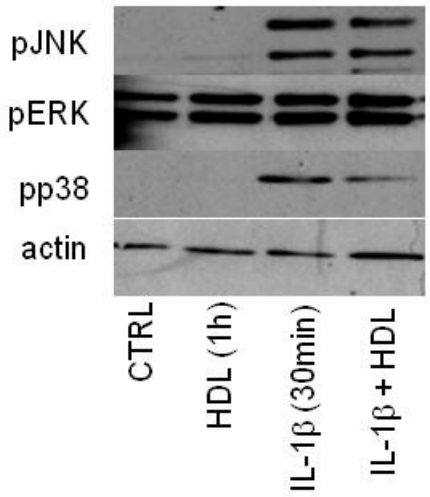


Figure 17: HDL down regulates p38 phophsorylation. Mouse islets were incubated for 1 hour with HDL alone or 30 min with HDL alone and subsequently 30 min with HDL and IL-1β together. Proteins were extracted and phosphorylation of MAP kinases was analysed by western blot with specific antibodies.

In an attempt to uncover which receptor is involved in the protection of β-cells by HDL against IL-1β induced apoptosis, mouse islets obtained from SRB1 -/- animals were exposed for 2 days to IL-1β alone or in the presence of HDL. Islets obtained from wild type littermates were exposed to the same conditions and used as controls. The islets were analysed for apoptosis by confocal microscopy, allowing the identification of apoptotic β-cells (insulin positive) and non-β-cells (insulin negative). HDL protected β-cells from IL-1β induced apoptosis in wild type islets as well as in SRB1 -/- islets (fig 18A+B). However, IL-1β induced less apoptosis in SRB1 -/- β-cells than in wild type β-cells. This makes any conclusion on the role of SRB1 in the mediation of HDL effect difficult to interpret.

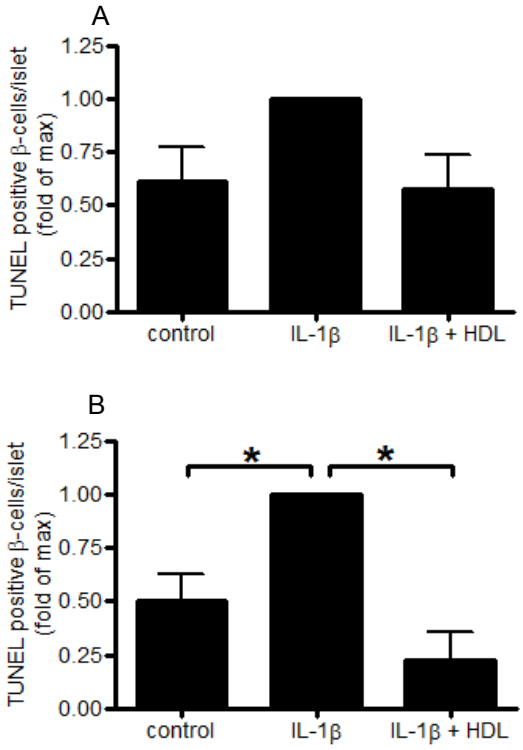


Figure 18: HDL protection of mouse β-cells from IL-1β induced apoptosis is independant from SRB1. Mouse islets from wild type and SRB1 -/- were cultured for 2 days on ECM-coated dishes in the absence (control) or presence of IL-1β (2 ng/ml) and in the absence (control) of presence of HDL (0.8 mM). A: TUNEL positive β-cell per SRB1 -/- islet (n=3), control (absolute value): 0.48 positive cells/islet. B: TUNEL positive β-cell per wild type islets (n=3), control (absolute value): 0.32 positive cells/islet. Where * represents $p<0.05$ as tested by ANOVA followed by Bonferonni's post-hoc test

5.2 Role of LDL on islet cells function, survival and apoptosis

5.2.1 Receptor expression

In order to evaluate the expression of the LDL receptor, a conventional PCR was performed on cDNA obtained from Min6 cells and human islets and on cDNA from purified human β-cells with primers specific for these receptors. In agreement with previous studies LDL receptor transcripts could be detected in Min6 cells and human

islets (fig 19A-C). However, LDL receptor transcripts could not be detected in purified human β-cells.

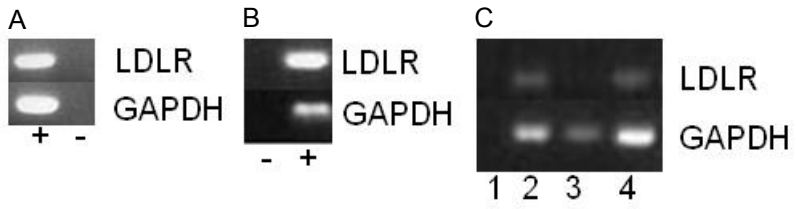


Figure 19: LDL receptors mRNA expression in human and mouse islet cells. Min6 cells, human islets, and purified human β-cells were cultured for 4 days on ECM-coated dishes and RNA was extracted. Conventional PCR with specific primers was performed to detect the expression of the LDL receptor. A: Min6 cells. B: Human islets. C: Purified human β-cells. (+) and (-) represent cDNA and RT negative control respectively. (1): negative control; (2) total islet cells; (3): β-cell fraction; (4): non-β-cell fraction

5.2.2 LDL and islet cells function

Since the LDL receptor is expressed in islet cells and was shown to be functional, the effects of LDL on islet function were investigated. Human and mouse islets were incubated for 4 days with LDL and glucose-stimulated insulin secretion was assessed. In both mouse and human islets, LDL decreased the stimulation of insulin secretion by glucose (fig 20A+B).

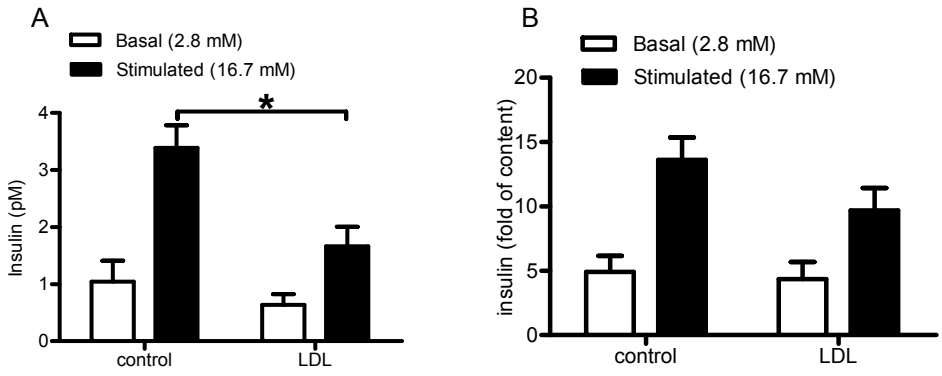


Figure 20: LDL decreases the glucose-stimulated insulin secretion in mouse and human β-cells. Mouse and human islets were cultured for 4 days on ECM-coated dishes in the absence (control) or presence of LDL (3.1 mM). A: Glucose-stimulated insulin secretion in mouse β-cells (n=5). B: Glucose-stimulated insulin secretion in human β-cells (n=6). * represents $p<0.05$ by Student's t test.

To investigate whether the LDL receptor was involved in the impairment of glucose-stimulated insulin secretion induced by LDL, isolated mouse islets from LDL receptor -/- mice, as well as from wild type mice, were cultured for 4 days in the presence of LDL prior to the assessment of glucose-stimulated insulin secretion. The impairment of glucose-stimulated insulin secretion was observed in the wild type β-cells, similar to what was obtained previously (fig 20A+21A), whereas this impairment was not observed with islets not expressing the LDL receptor (fig 21B). These data suggested that the impairment of glucose-stimulated insulin secretion due to LDL was dependent on functional LDL receptor.

Given the impaired glucose-stimulated insulin secretion induced by LDL, we investigated whether LDL affected the production of insulin in islet cells. After 4 days incubation with LDL, islet insulin content and insulin mRNA were measured. LDL appeared to impair only the stimulation of insulin secretion since no difference was observed in insulin content and insulin gene expression in mouse islets from LDL receptor -/- and LDL receptor +/+ islets (fig 21C-F).

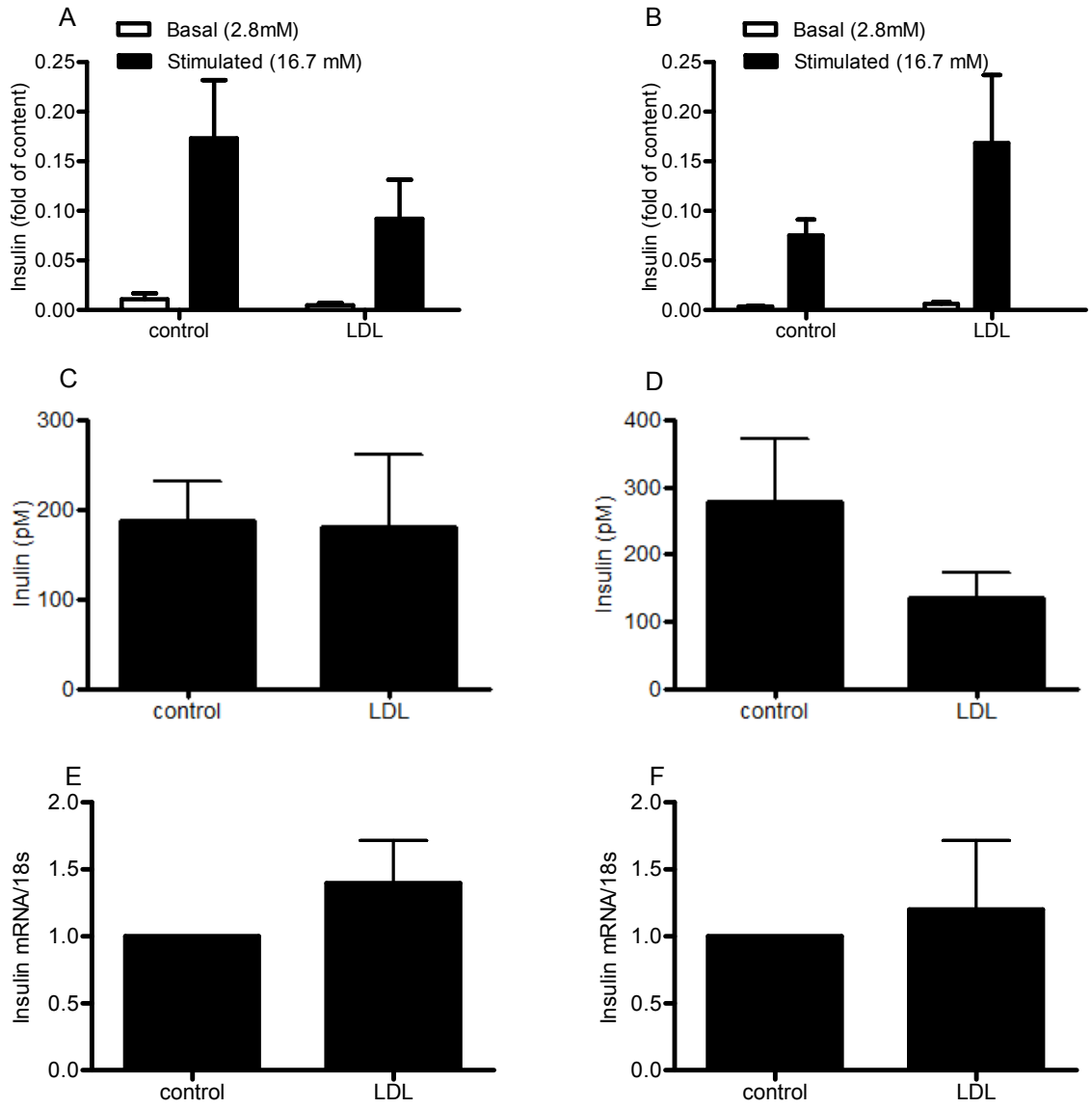


Figure 21: The LDL receptor mediates the functional impairment of islet cells induced by LDL. Mouse islets from LDR +/+ (A, C, E) and LDLR -/- (B, D, F) animals were cultured for 4 days on ECM-coated dishes in the absence (control) or presence of LDL (3.1 mM). A: Glucose-stimulated insulin secretion in LDLR +/+ mouse β-cells (n=3). B: Glucose-stimulated insulin secretion in LDLR -/- mouse β-cells (n=3). C: Total insulin content of LDLR +/+ islets (n=3). D: Total insulin content of LDLR-/- islets (n=3). E: Total insulin mRNA in LDLR+/+ islets (n=8). F: Total insulin mRNA in LDLR -/- islets (n=2).

5.2.3 LDL and islet cells proliferation

In order to investigate the role of LDL on islet cells proliferation, human and mouse islets were incubated in the presence of LDL for 2 and 4 days. After 4 days incubation, LDL decreased basal mouse and human islet cells proliferation up to 2

fold (fig 22B+D). This anti-proliferative effect could already be observed after 2 days in mouse islets (fig 22A). When LDL is incubated for 4 days in the presence of cells and glucose (5.5 mM for human and 11mM for mouse islets), the chances of LDL to be modified are elevated. In order to avoid a large increase in LDL modification, media and LDL were added freshly every day for 4 days. After 4 days, LDL decreased proliferation of islet cells (fig 22C). However, liraglutide, a GLP-1 analogue known to increase islet cells proliferation, was able to counteract the anti-proliferative effects of LDL on mouse islet cells (fig 23A). When LDL and media were changed every day during the incubation time (fig 23B) the same effects were observed. Thus, the anti-proliferative effects were strong enough not only to affect the basal but also the liraglutide induced islet cells proliferation. To determine which islet cell type is affected by LDL, single cells from isolated mouse islets were cultured for 4 days in the presence of LDL allowing us to distinguish between proliferative β-cells and proliferative non-β-cells. Indeed, LDL decreased specifically β-cell proliferation (fig 24).

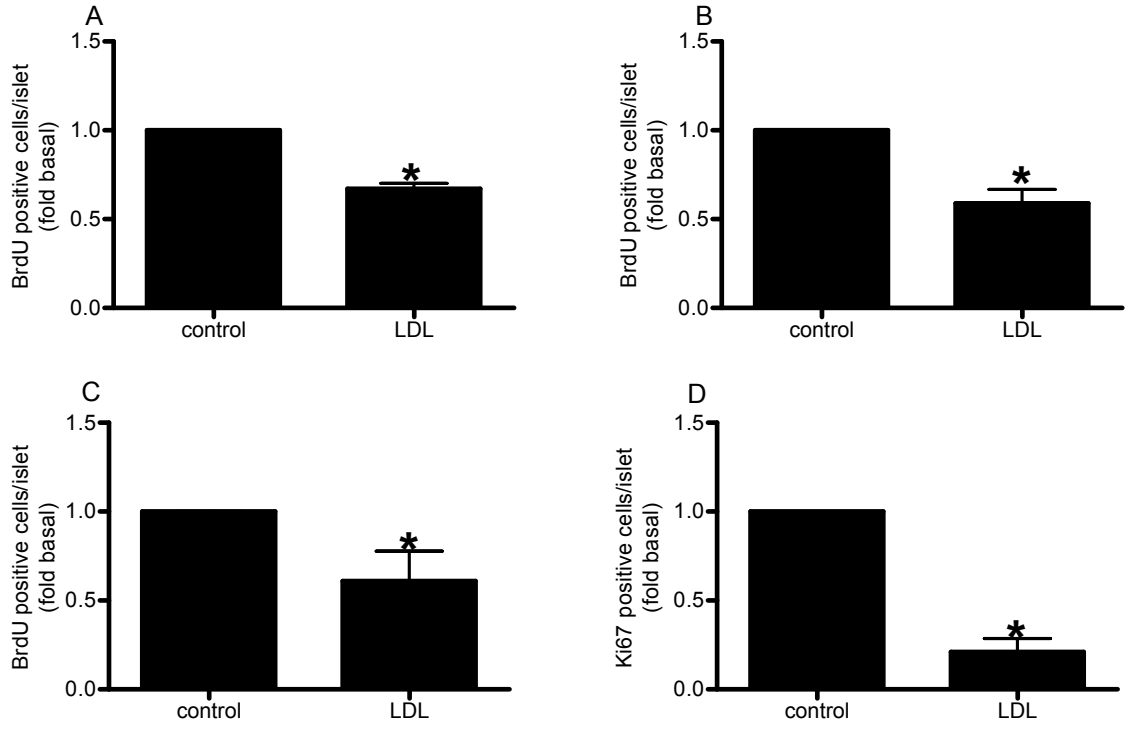


Figure 22: LDL decreases basal mouse and human islet cells proliferation. Mouse and human islets were cultured for 2 (A) or 4 (B-D) days on ECM-coated dishes in the absence (control) or presence of LDL (3.1 mM). A: BrdU positive mouse islet cells per islet after 2 days (n=3), control (absolute value):

3.87 positive cells/islet. B: BrdU positive mouse islet cells per islet after 4 days (n=9), control (absolute value): 3.09 positive cells/islet. C: BrdU positive mouse islet cells per islet after 4 days, with media changed and LDL freshly added every day (n=6), control (absolute value): 5.63 positive cells/islet. D: Ki-67 human islet cells per islet after 4 days (n=4), control (absolute value): 0.12 positive cells/islet. * represents $p<0.05$ by Student's t test.

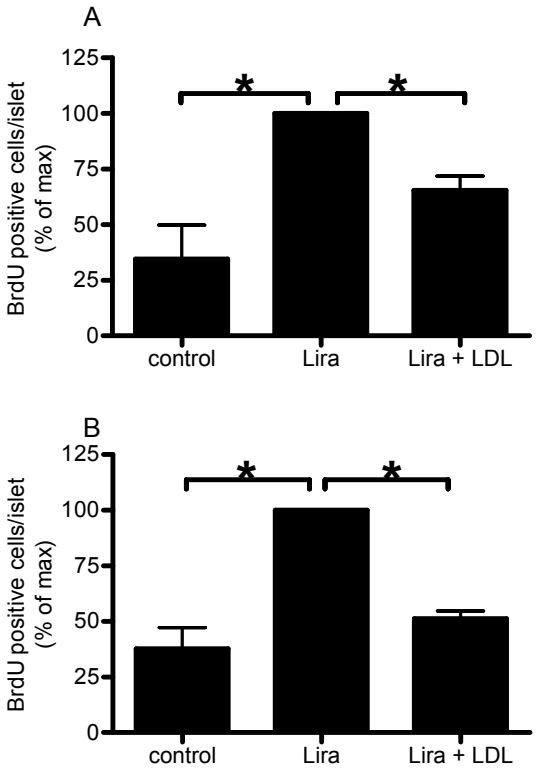


Figure 23: LDL decreases liraglutide induced mouse islet cells proliferation. Mouse islets were cultured for 4 days on ECM-coated dishes in the presence or absence (control) of liraglutide (Lira 1 mM) and in the absence (control) or presence of LDL (3.1 mM). A: BrdU positive mouse islet cells per islet after 4 days (n=3), control (absolute value): 1.95 positive cells/islet. B: BrdU positive mouse islet cells per islet after 4 days with media changed and LDL freshly added every day (n=6), control (absolute value): 5.64 positive cells/islet. Where * represents $p<0.05$ as tested by ANOVA followed by Bonferonni's post-hoc test.

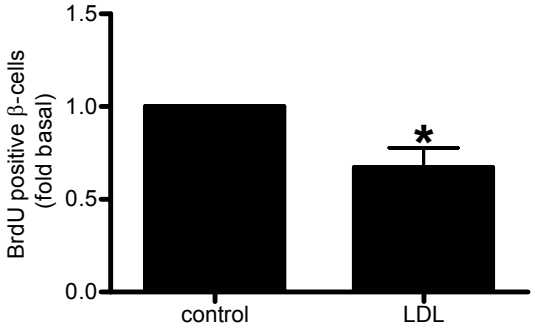
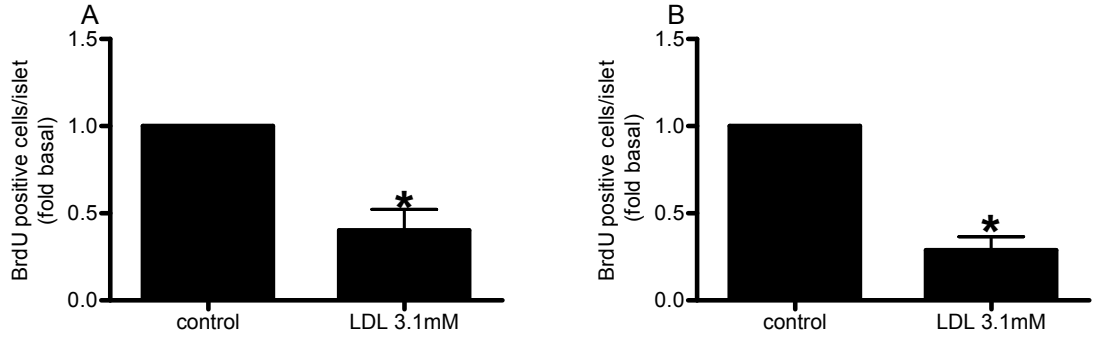


Figure 24: LDL decreases mouse β-cell proliferation. Single mouse islet cells were cultured for 4 days on ECM-coated dishes in the absence (control) or presence of LDL (3.1 mM). A: BrdU positive mouse β-cells after 4 days (n=6). Where * represents $p<0.05$ as tested by Student's t test.

To investigate whether LDL receptor was involved in the decrease of islet cells proliferation, islets isolated from LDL receptor -/- mice and control LDLR +/+ mice were incubated for 4 days with LDL and in the presence of liraglutide. The results obtained with LDLR -/- islets (fig 25B+D) were similar to those obtained with LDLR +/+ islets (fig 25A+C), where LDL decreased both basal and liraglutide induced proliferation, thus indicating that LDL receptor mediated LDL uptake was not involved in the anti-proliferative effect of LDL.



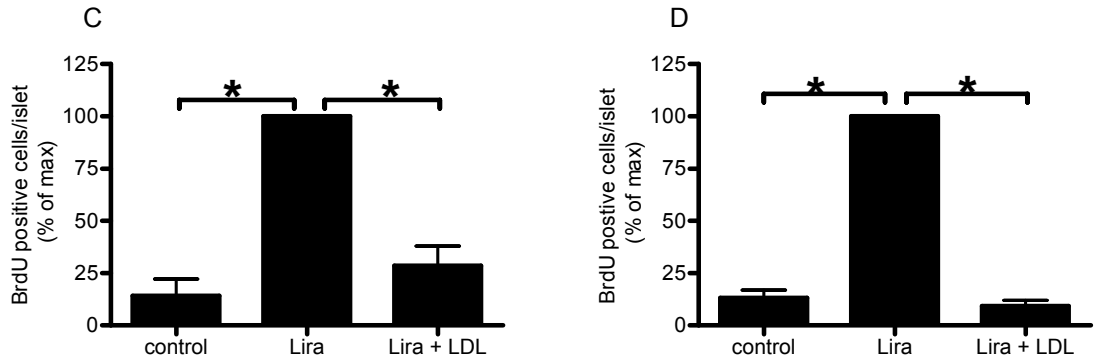


Figure 25: LDL decreased islet cells proliferation is independent of functional LDL receptor. Mouse islets from LDLR +/+ (A, C) or LDLR -/- (B, D) mince were cultured for 4 days on ECM-coated dishes in the presence or absence (control) of liraglutide (Lira 1 mM) and in the absence (control) or presence of LDL (3.1 mM). A: BrdU positive mouse islet cells per islet (n=3), control (absolute value): 1.06 positive cells/islet. B: BrdU positive mouse islet cells per islet (n=3), control (absolute value): 2.33 positive cells/islet. C: BrdU positive mouse islet cells per islet (n=3), control (absolute value): 1.06 positive cells/islet. D: BrdU positive mouse islet cells per islet (n=3), control (absolute value) 2.33 positive cells/islet. Where * represents $p<0.05$ as tested by ANOVA followed by Bonferonni's post-hoc test (C, D) or Student's t test (A, B).

5.2.4 LDL and islet cells apoptosis

Apoptosis of mouse and human islet cells was examined after 4 days incubation of the islets in the presence of LDL. LDL did not affect mouse or human islet cells apoptosis (fig 26A+B).

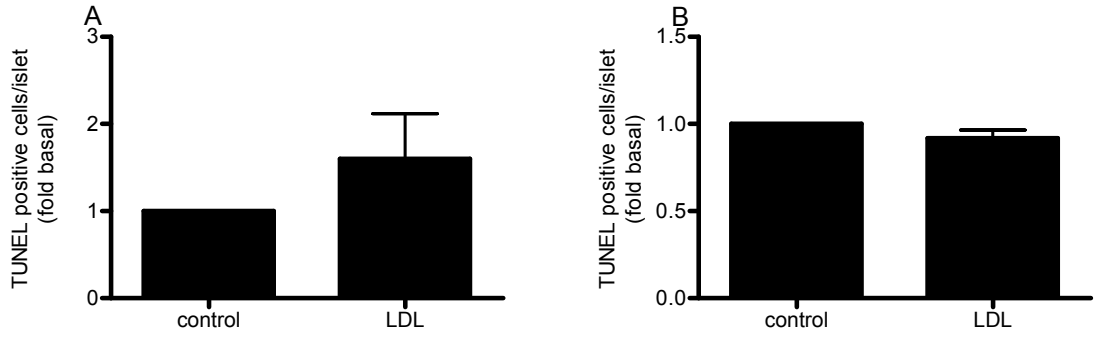


Figure 26: LDL does not affect islet cells apoptosis. Mouse (A) and human (B) islets were cultured for 4 days on ECM-coated dishes in the absence (control) or presence of LDL (3.1 mM). A: TUNEL positive mouse islet cells per islet (n=3), control (absolute value): 0.68 positive cells/islet. B: TUNEL positive human islet cells per islet (n=3), control (absolute value): 0.34 positive cells/islet.

6 DISCUSSION

Patients with type 2 diabetes typically display, along with hyperglycaemia, elevated circulating levels of free fatty acids and triglycerides and small dense LDL particles as well as low plasma levels of HDL cholesterol [121]. Even more so, low HDL cholesterol, hypertriglyceridemia and small dense LDL often precede the manifestation of diabetes mellitus type 2 and epidemiological studies unravelled low HDL cholesterol as an independent risk factor for developing type 2 diabetes [125]. In the past this association was explained as an innocent bystander effect of insulin resistance in the pre-diabetic state which interferes with several steps in HDL metabolism and thereby decreases HDL cholesterol either directly or by increasing free fatty acid levels. Most recent preliminary data in rodent β-cells have indicated a role for lipoproteins in the regulation of islet cell survival and insulin synthesis suggesting a causal or modulatory role of lipoproteins in the precipitation of diabetes mellitus type 2 [73, 128]. The present thesis has examined the role of HDL and LDL on mouse and human pancreatic β-cell apoptosis, proliferation and secretory function. The putative protective role of HDL on islet cells in the context of type 2 diabetes has been investigated. Finally, with this work we have tried to uncover the mechanisms of action of HDL and LDL on islet cells.

6.1 Role of HDL on β-cell function, proliferation and apoptosis

Plasma concentrations of lipoproteins are tightly controlled and are routinely evaluated in patients with diabetes. Concentrations of HDL > 1 mM and of LDL < 3.1 mM are considered within the normal range. Diabetic patients have often a dyslipidemia characterized by reduced plasma concentration of HDL. Total LDL plasma concentration is not changed in diabetic patients, however the sub-composition of LDL particles is changed, with increasing amount of small dense LDL that are more prone to modifications such as glycosylation or oxidation [120, 121, 124]. The concentrations used to treat the human and mouse islets in the present study are within these ranges. However, the precise concentration of lipoproteins around the islets is not known. Nevertheless, since the islets are highly vascularized

and islet endothelium fenestrated, we assumed that the lipoprotein concentration in the plasma around the islets is similar to the one in the circulation.

HDL has long been recognized as having a protective role on the arterial cell wall [132]. For example and in addition to its classical role in reverse cholesterol transport, HDL and its protein and lipid components exert anti-apoptotic and anti-inflammatory effects on endothelial and smooth muscle cells [67, 133]. This protective role of HDL has also been studied in murine islet cells and in the βTC3 β-cell line, where HDL had a protective effect on apoptosis induced by either a cocktail of cytokines, starvation or elevated LDL [73].

In the present work, we found that HDL decreases basal islet cells apoptosis without influencing the function or the proliferation of these cells. Moreover, HDL protected islet cells from glucose and IL-1β induced apoptosis. These protective effects were specific for β-cells as indicated by our immunological colocalization experiments. Furthermore, we showed that HDL influences human islet cells survival, suggesting relevance for human disease. The protective effects exerted by HDL on islet β-cells are an interesting finding suggesting an important role for this particle in the survival of these cells and particularly in the context of type 2 diabetes. However, it stills remains to be determined if HDL directly acts on β-cells or if the protective effect is mediated by another intermediary cell type, for example macrophages which were found to accumulate in pre-diabetic islets and to secrete potentially β-cell-toxic cytokines [51]. HDL in turn is known to strongly influencing macrophage activation including inhibiting cytokine secretion.

In an attempt to uncover the pathway by which HDL affects islet cells survival, HDL was separated into its protein and lipid moieties and both were investigated separately for their putative anti-apoptotic activity. The protein moiety of HDL displayed a trend to protect islet cells against IL-1β and glucose induced apoptosis. Moreover, the protective effect of purified ApoA1 against IL-1β induced apoptosis was statistically significant. Interestingly ApoA1 was previously found to exert similar anti-inflammatory effects also on endothelial cells [90].

Interestingly the lipid moiety of HDL protected islet cells from IL-1β induced apoptosis. Based on previous studies, which identified S1P as an inhibitor of apoptosis in endothelial cells and β-cells from apoptosis [81, 103, 105], we also tested the anti-apoptotic effect of S1P. However, at a concentration of 100 nM S1P

only showed a statistically not significant trend to protect mouse islet cells from IL-1β induced apoptosis. The discrepancy might be explained by the 10 to 1000 times lower S1P concentrations used here.

In order to uncover the signaling pathways by which HDL supports β-cell survival, we investigated the effects of HDL on signals which are known to mediate the apoptotic effects of IL-1β, namely induced iNOS and Fas expression [134]. We observed that HDL decreases Fas mRNA expression and increases FLIP mRNA expression. Prior to the Fas and FLIP regulation which appeared after 4 days, HDL first down regulated iNOS mRNA expression. In β-cells, up regulation of iNOS increases NO production. In turn, it is well established that NO, in β-cells, regulates a set of genes that are involved in apoptosis and specifically Fas [49]. Our results indicate that HDL interferes with the upregulation of iNOS and Fas mRNA expression by IL-1β. The expression of other genes involved in apoptosis, such as Bcl2 or SOCS3, was not influenced by HDL. MAP kinases, which are downstream effectors of IL-1β signaling, are known to regulate iNOS expression [47]. HDL decreased p38 phosphorylation induced by IL-1β, while HDL did not have any effect on JNK and ERK phosphorylation.

Of note, in endothelial cells HDL also interferes with apoptotic signals by altering NO production. However in this case, HDL induces the phosphorylation of endothelial nitric oxide synthase (eNOS) and thereby increases NO production which is anti-apoptotic in endothelial cells. [135]. Both S1P receptors and the HDL receptor SRB1 have been shown to mediate the anti-apoptotic effects of HDL in endothelial cells [75]. While the role of S1P must be further investigated, we tend to exclude the involvement of SRB1 in HDL islet cells protection against IL-1β by finding the protective effects of HDL on IL-1β induced β-cell apoptosis also in islets of SRB1 -/- mice. However, the results obtained in islets of SRB1 -/- mice must be interpreted with caution since the apoptosis induced by IL-1β in these islets is milder (1.5 x) compared to the apoptosis induced by IL-1β in islets from wild type mice (2 x).

Overall we hypothesize (fig 28) that HDL influences positively β-cell survival by down regulating iNOS and Fas mRNA expression. In parallel, HDL increases FLIP which may offer an additional protective effect. Upstream from iNOS regulation, other intracellular signaling proteins are probably regulated by HDL. In fact, HDL seems to

influence p38 phosphorylation and so decreasing the proapoptotic effect of IL-1β. ApoA1 protects islet cells from IL-1β induced apoptosis, similarly to HDL.

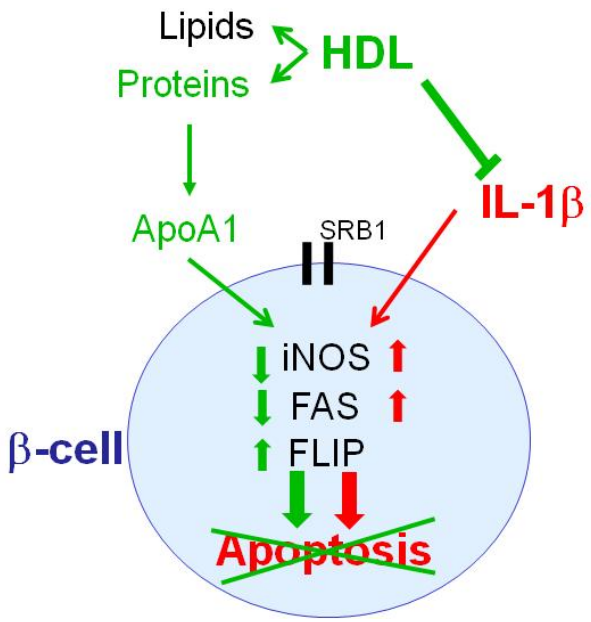


Figure 27: IL-1β, by inducing iNOS and Fas expression induces β-cell apoptosis. HDL, probably via its protein part, and specifically ApoA1, decreases Fas and iNOS, and increases FLIP mRNA expression thus resulting in a decrease in apoptosis. Moreover, HDL counteracts the pro-apoptotic effect of IL-1β on β-cells, independently of SRB1. In red: IL-1β effects on β-cells that result in apoptosis. In green: HDL effects on β-cells that increas cell survival

6.2 Role of LDL on β-cell function, proliferation and apoptosis

Consistent with previously published results, we observed LDL receptor expression in human and mouse islets, and on Min6 cells [73, 131]. However, no LDL receptor transcripts could be detected in purified human β-cells. The presence of LDL receptor in human β-cells has never been directly investigated. Human β-cells were shown to bind and internalize LDL particles [129, 131]. In other cell type, the major entrance door for LDL to the cells is the LDL receptor. However, it was never directly investigated if the LDL receptor is responsible for binding and uptake of the LDL particles observed in human β-cells.

In this study we found that LDL decreases glucose-stimulated insulin secretion in mouse and human islets. The LDL receptor appears to play a central role in this functional impairment, since glucose-stimulated insulin secretion in islets obtained from the LDL receptor -/- mice was not influenced by the presence of LDL. We further hypothesize that LDL only affects the secretory machinery and not the production of insulin since no difference was observed in total insulin content or in insulin mRNA level of LDL treated and LDL untreated islets of either wild type mice or LDL receptor -/- mice. Since the LDL receptor acts as an endocytic receptor we propose that the impairment of glucose-stimulated insulin secretion by LDL is mediated by a component of LDL which is internalized by the LDL receptor pathway. Most likely this component is cholesterol because impaired cholesterol homeostasis is known to contribute to islet dysfunction. The impairment of insulin secretion that follows the accumulation of cholesterol into the β-cells is a consequence of a defect in the secretory machinery and not of a defect in the insulin production [128]. With this work, we show that LDL, probably by causing cholesterol accumulation in the islet cells, impairs glucose-stimulated insulin secretion in β-cells without influencing the insulin production.

In parallel to the impairment of the secretory function of β-cells, LDL also decreased the basal proliferation of mouse and human islet cells. However, in contrast to the effect on insulin secretion, this anti proliferative effect appears to be independent of LDL uptake by the LDL receptor, since islets from LDL receptor -/- mice were found as sensitive as wild type islets to the decrease in proliferation induced by LDL. This implicates that LDL effects on proliferation are probably independent of intracellular cholesterol accumulation, since the LDL receptor is the major entrance door for cholesterol. This anti-proliferative effect of LDL on β-cells contrasts with previously published studies where LDL rather induced proliferation of smooth muscle cells [136], suggesting that the effects of LDL on the cell cycle is dependent on the cell type. A possible mediator of the anti-proliferative effect of LDL on β-cells could be apoER2, which is expressed on β-cells and which contributes to LDL signaling on platelets [73, 137]. However, other members of the LDL receptor family should not be neglected when investigating the pathway mediating the decrease in β-cells proliferation induced by LDL. Finally, the decrease in proliferation could be reversed

by treating the cells in parallel with liraglutide, a GLP-1 analogue known to increase proliferation.

The effects of LDL on islet cells survival are not clear. One study observed that elevated LDL induces apoptosis of islet cells and a murine β-cell line [73]. However, Abderrahmani and collegues could not reproduce these effects using lower LDL concentrations, and they observed that LDL induces apoptosis only when it is oxidized and not in its native form [118]. In the present work, mouse and human islet cells survival was not influenced by the presence of low concentrations of LDL, suggesting that LDL probably increases apoptosis of the islet cells only at elevated concentrations or when it is modified by oxidation.

7 CONCLUSION AND FUTURE DIRECTIONS

The present thesis was undertaken to elucidate the role of HDL and LDL on mouse and human pancreatic islet β-cells secretory function, proliferation and apoptosis. The present work proposes that HDL protects basal islet cells apoptosis in addition to protecting the cells from glucose and IL-1β induced apoptosis. This work furthermore suggests that plasma concentrations of LDL should be tightly regulated since prolonged exposure to LDL impairs β-cell insulin secretion and decreases islet cell proliferation. These deleterious effects can be partially prevented by the GLP-1 analogue liraglutide. It is hoped that this thesis has provided some useful insights into the possible importance of well controlled LDL and HDL plasma concentrations to prevent the progressive decline in β-cell mass responsible for the onset and progression of type 2 diabetes. Furthermore it is hoped that this knowledge will contribute to understand why low HDL concentration is a risk factor for developing type 2 diabetes.

The main conclusions from this thesis are:

HDL decreases basal mouse and human islet cells apoptosis.

HDL protects β-cells from IL-1β and glucose induced apoptosis, probably independently of SRB1.

ApoA1 mimic the protective effect of HDL.

The anti-apoptotic effects of HDL may be mediated via decreased iNOS and Fas, and increased FLIP expression.

LDL impairs glucose-stimulated insulin secretion, dependent on functional LDL receptor.

LDL decreases basal proliferation in mouse and human islets, an effect that is partly prevented by liraglutide.

8 MATERIEL AND METHOD

8.1 Reagents

Recombinant mouse and human IL-1β were obtained from R&D (R&D systems, Minneapolis, USA). Sphingosine-1-phosphate was obtained from Avanti (Avanti polar lipids, Alabaster, USA) and reconstituted in methanol. Human isolated ApoA1 was purchased from Calbiochem (Calbiochem, Gibbstown, USA).

8.2 Cells

8.2.1 Mouse islets isolation

Mouse islets were isolated from C57BL/6J mice, LDL receptor -/- mice (Jackson Laboratory, USA), and SR-B1 -/- mice (kindly provided by G.Georgopoulos, Athens, Greece). The islets were isolated by collagenase digestion (Worthington Biochemical Corporation, Lakewood, USA) of the pancreas and separated from the exocrine tissue by density gradient (Histopaque-1119, Sigma-Aldrich, USA). Islets were further handpicked prior to plating for higher purity.

8.2.2 Mouse islets culture

Mouse islets were cultured in RPMI-1640 medium containing 11 mmol/l glucose, 40 μg/ml gentamycin, 100 units/ml penicillin, 100 μg/ml streptomycin, 1% glutamax and 10% FCS (Invitrogen, Basel, Switzerland). Islets were cultured on ECM-coated plates derived from bovine corneal endothelial cells (Novamed, Jerusalem, Israel). The islets were cultured in islet media for 48h to adhere to the ECM-coated plate and to spread prior to the initiation of the experiments. Islets were treated with 22 mmol/l or 33 mmol/l glucose, recombinant mouse IL-1β (R&D, Minneapolis, USA), human lipoproteins, apolipoprotein A-1 (Calbiochem, San Diego, USA) or sphingosine-1-phosphate.

8.2.3 Human islets culture

Human islets were isolated from pancreta of organ donors at the University of Geneva Medical Centre (Geneva, Switzerland) and at the INSERM EIT-M 0106 in Lille (Lille, France). Human islets were cultured in CMRL-1066 medium containing 5 mmol/l glucose, 100 units/ml penicillin, 100 μg/ml streptomycin, 1% glutamax and 10% FCS (Invitrogen, Basel, Switzerland). Islets were cultured on ECM-coated plates derived from bovine corneal endothelial cells (Novamed, Jerusalem, Israel). The islets were cultured in islet media for 48h to adhere to the ECM-coated plate and to spread prior to the initiation of the experiments. Islets were treated with 33 mmol/l glucose, recombinant human IL-1β (R&D, Minneapolis, USA) or human lipoproteins.

8.2.4 Mouse single islet cells culture

To obtain single islet cells, mouse islets freshly isolated were washed twice in Mg^{2+} and Ca^{2+} -free phosphate-buffered saline (PBS: 80g NaCl, 2g KCl, 11.5 g Na_2HPO_4 $2H_2O$, 2.5 g KH_2PO_4 in 10 litre H_2O) + 0.5 mM EDTA. Thereafter, islets were incubated for 6 minutes at 37°C in 600 μl trypsin-EDTA (Invitrogen, Basel, Switzerland) and 900 μl Mg^{2+} and Ca^{2+} -free PBS + 0.5 mM EDTA. To ensure single cells, the islets were mechanically dispersed by pipetting and culture media was added to stop the trypsinisation. Cells were centrifuged (1000 rpm for 3 minutes), resuspended in culture media, and plated on ECM-coated plates for culture.

8.2.5 Cell lines culture

The murine insulinoma cell line Min6 was cultured in regular cell culture dishes in DMEM medium containing 25 mM glucose and glutamine completed with 40 μg/ml gentamycin, 100 units/ml penicillin, 100 μg/ml streptomycin, 15% FCS and 0.1% β-mercaptoethanol (Invitrogen, Basel, Switzerland)

The rat insulinoma cell line INS1E was cultured in regular culture dishes in RPMI-1640 medium containing 11 mmol/l glucose, 40 μg/ml gentamycin, 100 units/ml penicillin, 100 μg/ml streptomycin, 1% glutamax, 10 mM Na pyruvate, 0.1% glutamax, 10 mM β-mercaptoethanol and 10% FCS (Invitrogen, Basel, Switzerland).

8.3 Lipoproteins

8.3.1 Isolation of human lipoproteins

Blood was collected from healthy donors. Plasma low density lipoprotein (LDL) and high density lipoprotein (HDL) fractions were isolated by sequential ultracentrifugation (LDL: 1.006 < LDL density < 1.063; HDL: 1.063 < HDL density < 1.21), dialyzed against LDL-buffer (NaCl 1.5 M, EDTA 3 mM, pH 7.4) and finally filter sterilized using a 0.22 μm microfilter. Cholesterol concentration was measured using an enzymatic test. The purity of the lipoprotein preparation was verified by apolipoprotein composition analysis in a 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) to exclude contaminations with LDL or albumin in the HDL fraction and to exclude contamination with HDL or albumin in the LDL fraction. Lipid-free HDL protein moiety was further extracted from HDL as described previously [138].

8.3.2 Preparation of reconstituted HDL

Discoidal reconstituted HDL (rHDL) was produced out of a complex containing 1-palmitoyl-2-oleyl-L-palmitoyl-sn-glycero-3-phosphatidylcholine (POPC) (Fluka), lipid – free HDL protein moiety and sodium cholate (NaCholate) in a molar ratio of 100/1/100. In brief 2.71 mg of POPC was dissolved in CHCl3, added to a conical glass tube and dried under nitrogen. Thereafter, POPC was dissolved in 0.4 ml Tris saline (0.01 M Tris, 0.15 M NaCl, 0.005 mM EDTA), pH8. The mixture was vortexed thoroughly and stored on ice for 1 hour. 100 μl NaCholate (from 157 mg/ml stock solution) was added and the mixture was then vortexed every 10 min for 75 min until completely clear. After clearing, 1 mg of lipid-free HDL protein moiety in Tris saline was added and the mixture was then incubated for 1 hour on ice. Finally the reconstituted HDL was extensively dialysed at 4°C in Tris saline.

8.3.3 Extraction of the HDL lipid fraction

Human HDL was further processed to extract its lipid fraction. HDL was diluted in H_2O to obtain 1 ml of solution and the pH was adjusted to 3.0 with sulphuric acid (H_2SO_4). A biphasic (aqueous/organic) mixture was then formed by the addition of 1 ml of acetonitril (C_3H_3N) to the diluted acidic HDL. 0.5 g NaCl was also added. The

mixture was centrifuged at 800 g for 5 min at room temperature. The upper organic phase, containing the HDL lipid fraction, was transferred in a new vial and lyophylised. The lipid fraction was resuspend in ethanol and rapidly injected in PBS containing 1% fatty acid free BSA while vortexing.

8.4 RNA isolation and RT-PCR

8.4.1 Total RNA isolation

Total RNA was isolated from 100 mouse or human islets plated on ECM-coated culture dishes with a total RNA extraction kit (NucleoSpin RNAII, Marcherey Nagel, Düren, Germany). The cells were harvested by scraping them off the plate in the RA1 buffer provided with the RNA extraction kit. The lysed cells were transferred to the purification column and RNA was further extracted according to the manufacturer's instruction.

8.4.2 Reverse transcription

First strand cDNA synthesis was performed using 1 μl (200U) SuperScriptTM II RT (Invitrogen, Basel, Switzerland). Typically 3 μl dTT, 2 μl mixed dNTPs (10 mM each), 12 μl 5x First-Strand Buffer, 1 μl random hexamers, 1 μl Supercript and 0.5 μl RNase inhibitor were mixed with 40 μl RNA and incubated at 37°C for 2 hours. The enzymatic reaction was stopped by incubating the samples at 70°C for 15 min. After a quick spin down, the cDNA samples were stored at -20°C.

8.4.3 Polymerase chain reaction

The conventional polymerase chain reaction (PCR) was performed with cDNA samples obtained from the reverse transcription. 2 μl cDNA were mixed with 25 μl TaqMan PCR Master Mix (Qiagen, Germantown, USA), 2 μl forward primer (10 μM), 2 μl reverse primer (10 μM) and 19 μl H_2O to obtain a final volume of 50 μl. Specific primer sequences and annealing temperatures are listed in tables 1 and 2. Typical cycling parameters were: 1 cycle 94°C for 1 min, 25 to 45 cycles 92°C 1 min + 52-56°C 1 min + 72°C 1 min and finally 1 cycle 72°C 7 min, performed with the

TGradient PCR machine (Biometra, Göttingen, Germany). The PCR products were analysed on a 2% polyacrylamide gel containing 0.006% ethidium bromide.

8.4.4 Semi-quantitative polymerase chain reaction

The TaqMan technology (Applied Biosystems, USA) was used as the semi-quantitative PCR method. The cDNA samples, obtained from the reverse transcription, were diluted 1/12 in H_2O prior to their loading on a 96-well reaction plate (MicroAmp, Applied Biosystems, USA). The specific primers containing the fluorescent probe were diluted 1/10 in Universal PCR Master Mix (TaqMan, Applied Biosystems, USA) and then added to the samples on the reaction plate. The specific TaqMan primers are listed in table 3. The detection of the amplification was performed with the 7500 Fast Real-Time PCR System (Applied Biosystems, USA).

8.5 Western Blot

8.5.1 Protein isolation and protein concentration measurement

The proteins were extracted from approximately 200 mouse islets plated on ECM-coated dishes. Following a washing step in 1 ml PBS, the islets were scrapped of the culture dish and put in a tube containing 1 ml PBS. After a centrifugation at 2000 rpm for 5 min at 4 °C, the supernatant was discarded and the pellet was respuspended in 100 µl of lysis buffer containing: 20 mM Tris (pH 7.5), 150 mM NaCl, 1% Triton X-100, 10% Glycerol, 10 µl Protease inhibitor cocktail (Roche, Basel, Switzerland), 1 mM Na_3VO_4, 1 mM NaF, 0.5 mM PMSF and 1 mM EDTA (pH 8) and then incubated for 15 min on ice. The lysate was sonicated and centrifuged at 13000 rpm for 10 min at 4°C. The supernatant containing the proteins was stored at -20°C. The concentration of the protein was measured with the conventional bicinchoninic acid (BCA) protein assay kit (Pierce, Rockford, USA).

8.5.2 Western Blotting

Equivalent amount of protein lysate (20-50 µg) were mixed with SDS sample buffer and denaturated at 70°C for 10 min and subjected to a 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Proteins were electrically transferred to nitrocellulose filter membranes. Blocking of non-specific protein binding

was done by incubating filter membrane in blocking buffer (1xTBS 3.03 g Tris-HCl, 8.7 g NaCl in 1 l H_2O (pH 7.6), 0.1% Tween-20 and 5% non-fat dry milk) for 1 hour. After washing in TBS-T (1 x TBS (pH 7.6), and 0.1% Tween-20), filters were incubated at 4°C overnight with the specific primary antibodies. Subsequently, filter membranes were washed in TBS-T and incubated for 1 hour with horseradish peroxidise-conjugated secondary antibodies. Immune complexes were detected by chemoluminescence using LumiGLO (Cell singaling technology, Danvers, USA) and light emission was captured on X-ray film.

8.6 Detection of Apoptosis

8.6.1 Fluorescence

Mouse and human islets on ECM-coated dishes were fixed with 4% paraformaldehyde for 30 min at room temperature. The cells were permeabilised with Triton-X for 4 min prior to their staining. The free 3'-OH strand breaks resulting from DNA degradation were detected by the terminal deoxynucleotidyl-transferase-mediated dUTP nick-end labelling (TUNEL) technique according to the manufacturer's instructions (In Situ Cell Death Detection Kit, Roche, Basel, Switzerland). Islets were co-stained for insulin with a guinea pig anti-insulin antibody (DakoCyomation, Glostrup, Denmark). The number of apoptotic cell was determined by a careful counting with a fluorescent microscope (Axioplan 2, Zeiss, Germany).

8.6.2 Confocal

Mouse islets were co-stained for apoptosis (TUNEL, as described above), insulin and their nucleus (4',6-diamidino-2-phenylindole, DAPI). Multiple labelling immunofluorescence images of mouse islets were captured using a scanning confocal head coupled to an upright microscope (TCS SP2, Leica, Wetzlar, Germany) with UV light and an Ar-Kr laser utilizing 358 nm, 488 nm and 647 nm excitation and a 63x/1.40 oil PlanApo objective lens. For each islet a series of eight pictures, corresponding to eight different z plans, was acquired with Leica software (Leica, Wetzlar, Germany) and merged. The series allowed the determination of the number of apoptotic β-cell and apoptotic non β-cell for each islet.

8.6.3 Cell line

The apoptosis in the different cell lines was ascertained using Cell Death Detection ELISA (Roche, Basel, Switzerland) following manufacturer's instructions. Because the lysis buffer contained in the ELISA assay did not allow the determination of the protein concentration with any method and though the cell number plated in each well was consistent, 2 identical 24-well plates for each experiment were treated and analysed in parallel. One was used for apoptosis determination and the other was used for protein concentration measurement. The supernatant was analysed with Bicinchoninic acid (BCA) Protein Assay kit (Pierce, Rockford, USA) to determine the protein concentration.

8.7 Detection of Proliferation

8.7.1 Ki-67 staining

For human islet cells proliferation studies, a monoclonal antibody against the human Ki-67 antigen (Zymed laboratories, San Fransisco, USA) was used. Islets were co-stained for insulin with a guinea pig anti-insulin antibody (DakoCyomation, Glostrup, Denmark). The number of proliferative cell per islet was determined by a careful counting with a fluorescent microscope (Axioplan 2, Zeiss, Germany).

8.7.2 BrdU staining

For mouse islet cells proliferation studies, BrdU was added to the mouse islets at the start of the treatment. The incorporated BrdU was detected with an anti-Brdu antibody (Roche, Basel, Switzerland). Islets were co-stained for insulin with a guinea pig anti-insulin antibody (DakoCyomation, Glostrup, Denmark). The number of proliferative cell per islet was determined by a careful counting with a fluorescent microscope (Axioplan 2, Zeiss, Germany).

8.8 Functional test

8.8.1 Glucose-stimulated insulin secretion

For acute insulin release in response to glucose, islets were washed and incubated in Krebs-Ringer buffer containing 2.8 mmol/l glucose and 0.5% BSA for 1h (= basal

secretion). The islets were then incubated in Krebs-Ringer buffer containing 16.7 mmol/l glucose and 0.5% BSA for 1h (= stimulated secretion). The total insulin content was obtained by measuring the insulin concentration in the supernatant of the islets incubated for 2 hours at room temperature with acid ethanol. Secreted insulin was assayed by radioimmunoassay (CIS Biointernational, Gif-sur-Yvette, France).

8.9 Statistical analysis

Data are expressed as means ± SEM, with the number of individual experiments presented in the figure legends. All data were tested for normality and analysed with PRISM (GraphPad, San Diego, USA). Significance was tested using the Student's t test and ANOVA with Bonferonni's post hoc test for multiple comparison analysis. Significance was set as $p<0.05$. All experiments were done at least in triplicates and considered as n=1.

8.10 Tables

Gene	Direction	Primer Sequence (5'-3')	PCR Annealing Temp (°C)
CD 36	Forward	TCGGAACTGTGGGCTCATTG	55
	Reverse	CCTCGGGGTCCTGAGTTATATTTTC	
SRBI	Forward	GGAATCCCCATGAACTG	52
	Reverse	AGCCCTTTTTTACTACCACTCC	
ABCA1	Forward	TGCCCTATGTGCTGTGCGTAG	55
	Reverse	GGTGAGATTGAAGCCGTCCTC	
GAPDH	Forward	CGACCCCTTCATTGAC	55
	Reverse	TCCACGACATACTCAGCAC	

Table 1: Primers sequences for target gene amplification in mouse with conventional PCR.

Gene	Direction	Primer Sequence (5'-3')	PCR Annealing Temp (°C)
CD36	Forward	ATATGGTGTGCTAGACAT	55
	Reverse	CATCACCACCACCAACAC	
SRBI	Forward	GGAATCCCCATGAACTG	55
	Reverse	CAGGCAAGCGAATGGC	
ABCA1	Forward	GTCATTATCATCTTCATCTGCTTCC	55
	Reverse	CCTCACATCTTCATCTTCATCATTC	

Table 2: Primers sequences for target gene amplification in human with conventional PCR.

Gene	AssayID	Assay Location
Socs3	Mm01249143_g1	289
Bcl2	Mm00477631_m1	1978
Nos2	Mm01309902	1168
Fas	Mm00433237	76
Flip	Mm01255580	1780

Table 3: Primer references for target gene amplification in mouse with TaqMan real-time PCR (Applied Biosystems)

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